FORM PTO-1390	ATTORNEY DOCKET NUMBER
U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	PU-0082
	U.S. APPLICATION NO. (if known see 37 C.F.R. 1.5) **10/009228**

INTERNATIONAL APPLICATION NO. PCT/US00/12528	INTERNATIONAL FILING DATE 08 May 2000	PRIORITY DATE CLAIMED 10 May 1999

TITLE OF INVENTION **COMPOSITIONS AND METHODS FOR IMPROVING LEARNING AND MEMORY**

APPLICANT(S) FOR DO/EO/US **Joe Z. TSIEN**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. **X** This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. __ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. **X** This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. **X** A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. **X** A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
  a. __ is transmitted herewith (required only if not transmitted by the International Bureau).
  b. __ has been transmitted by the International Bureau.
  c. **X** is not required, as the application was filed in the United States Receiving Office (RO/US)
6. __ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. **X** Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  a. __ are transmitted herewith (required only if not transmitted by the International Bureau).
  b. __ have been transmitted by the International Bureau.
  c. __ have not been made; however, the time limit for making such amendments has NOT expired.
  d. **X** have not been made and will not be made.

8. __ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. __ An oath or declaration of the inventor(s) 35 U.S.C. 371(c)(4).

10. __ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

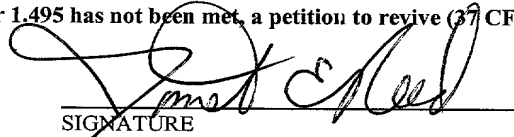**Items 11. to 16. below concern other document(s) or information included:**
11. __ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. __ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. __ A FIRST preliminary amendment.
  __ A SECOND or SUBSEQUENT preliminary amendment.

14. __ A substitute specification.

15. __ A change of power of attorney and/or address letter.

16. **X** Other items or information:
  - **Copy of the published PCT Application by WIPO under No. WO 00/67803, including the search report.**
  - **Copy of the International Examination Report.**
  - **Sequence Listing in written (1 sheet) and computer readable form.**
  - **Transmittal Letter submitting Sequence Listing and Statement to Support Filing and Submission.**

EXPRESS MAIL Mailing Label No.
Date of Deposit: **EL 899364615 US**
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231
MAILER_____ **John Hill**
SIGNATURE_____

EL899364615US

U.S. APPLICATION NO. (if known 37 CFR 1.5) **10/009228**	INTERNATIONAL APPLICATION NO. PCT/US00/12528	ATTORNEY DOCKET NUMBER PU-0082

17. _ The following fees are submitted:
Basic National Fee (37 CFR 1.492(a)(1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
and International Search Report not prepared by the EPO or JPO.........................$1,040.00

International preliminary examination fee (37 CFR 1.482 not paid to USPTO
but International Search Report has been prepared by the EPO or JPO...................$890.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but
international search fee (37 CFR 1.445(a)(2)) paid to USPTO................................$740.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) but
all claims did not satisfy provisions of PCT Article 33(1)-(4)...............................$710.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and
all claims satisfied provisions of PCT Article 33(1)-(4)...$100.00

CALCULATIONS   PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 100.00	
Surcharge of $130.00 for furnishing the oath or declaration later that _ 20 _ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total claims	49- 20 =	29	X $18.00	$ 522.00	
Independent Claims	9- 3 =	6	x $84.00	$ 504.00	
Multiple dependent claims(s) (if applicable)			+ $280.00	$	

TOTAL OF ABOVE CALCULATIONS =	$1,126.00	
_X_ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.	$ 563.00	
SUBTOTAL =	$ 563.00	
Processing fee of **$130.00** for furnishing the English translation later the _ 20 _30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$	
TOTAL NATIONAL FEE =	$ 563.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +		
TOTAL FEES ENCLOSED =	$ 563.00	
	Amount to be: refunded	$
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a. _X_ A check in the amount of $ **563.00** to cover the above fee is enclosed.

b. _ Please charge my Deposit Account No. 23-3050 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. _X_ The Commissioner if hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-3050. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

_____
Janet E. Reed
NAME

_____
36,252
REGISTRATION NUMBER

COMPOSITIONS AND METHODS FOR
IMPROVING LEARNING AND MEMORY

This application claims priority to U.S. Provisional Application No. 60/133,371, filed May 13, 1999, the entirety of which is incorporated by reference herein.

5

FIELD OF THE INVENTION

This invention relates to the field of neurobiology. In particular, the invention provides a method to improve learning and memory in humans and
10   animals, through the modification of the NMDA receptor.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application to more fully describe the state of the art
15   to which this invention pertains. The disclosure of each such publication is incorporated by reference herein.

It has been hypothesized that memory storage in the mammalian brain involves modifications of the synaptic connections between neurons. Hebb's rule (1949)
20   of "correlated activity" holds that both learning and memory are based on modifications of synaptic strength among neurons that are co-active, i.e., when presynaptic and postsynaptic neurons are active simultaneously, their connection becomes strengthened.

25   It has been postulated that N-methyl-D-aspartate (NMDA) receptors can implement the Hebb rule at the synaptic level. For this reason, they are considered good candidates as synaptic elements for the induction of activity-dependent synaptic plasticity. NMDA receptors

act as coincidence detectors because they require both presynaptic activity (glutamate released by axonal terminals) and postsynaptic activity (depolarization that releases the Mg^{2+} block) as condition for channel opening.
5 Active NMDA receptor channels allow calcium influx into the postsynaptic cell, which triggers a cascade of biochemical events resulting in synaptic change.

The NMDA receptors are heteromeric complexes consisting of NR1 and various NR2 subunits (Nakanishi,
10 *Science* **258**, 597-603, 1992; Hollmann & Heinemann, *Annu. Rev. Neurosci* **17**, 31-108, 1994). The NR1 subunit is essential for channel function, whereas the NR2 subunit regulates channel gating and Mg^{2+} dependency (Monyer et al., *Science* **256**, 1217-21, 1992). In the adult forebrain
15 regions such as the hippocampus and the cortex, only NR2A and NR2B subunits are available to form the receptor complex with NR1 subunit. The recombinant NR1-NR2B complex *in vitro* has a longer duration of excitatory postsynaptic potentials than those of the NR1-NR2A
20 complex (Monyer et al., *Neuron* **12**, 529-40, 1994). NR2B expression is down-regulated during the transition period from juvenile to adulthood (Sheng et al., *Nature* **368**, 144-147, 1994; Okabe et al., *J. Neurosci.* **18**, 4177-88, 1998), correlating with the gradual shortening of the
25 NMDA channel duration (Carmignoto & Vicini, *Science* **258**, 1007-11, 1992; Hestrin, *Neuron* **9**, 991-9, 1992).

The phenomena of long term potentiation (LTP) and long term depression (LTD) have been a focus of studies on neural plasticity at the molecular level.
30 These terms refer to multiple mechanisms involved in altering the strength of synapses. The induction of LTP requires, at least in one form, the activation of NMDA receptors. It is believed that NMDA receptor-dependent LTP is elicited by giving a strong pattern of electrical

stimulation (i.e., a 25-100 Hz train for ~1 sec) to the inputs, which triggers a rapid and lasting increase in synaptic strength.

5   The hippocampus is the most intensely studied region for the importance of NMDA receptors in synaptic plasticity and memory. It is known that lesions of the hippocampus in humans and other mammals produce severe amnesia for certain memories (see review of Squire, 1987). Disruption of NMDA receptors in the hippocampus

10   has been shown to lead to blockade of synaptic plasticity and also to memory malfunction (reviewed by Morris et al., 1991; Rawlins, 1996). For instance, application of NMDA receptor antagonists completely blocks the induction of LTP in most hippocampal synapses. Rats that received

15   infusion of such antagonists into the brain were deficient in performing certain spatial memory tasks (Morris et al., 1986). Similar results were observed in genetically engineered knockout mice which lacked a gene encoding a component believed to be downstream of

20   activated NMDA receptors in the biochemical cascade for LTP induction (Silva et al., 1992a; Silva et al., 1992b; reviewed by Chen & Tonagawa, 1997).

Although the foregoing results circumstantially implicate NMDA receptor-induced LTP in the hippocampus as

25   critical for certain types of memory and learning, they are equivocal, due to the non-specific nature of the respective treatments. For instance, in the case of the gene knockout mice, every cell in the animals lacks the gene of interest, thereby affecting all functions of the

30   gene product, not just LTP induction. Likewise, in pharmacological studies, the target of NMDA receptor antagonist infusion was not restricted to the hippocampus; therefore, NMDA receptors expressed in neurons of the neighboring neocortex and other brain

35   areas were also inhibited to a varying extent. Moreover,

this non-restricted binding has often been observed to produce sensory and motor disturbances.

To circumvent some of the interpretational difficulties arising from the lack of specificity in the aforementioned gene knockout or pharmacological results, a NMDA receptor knockout mouse was developed that comprised deletion of the NR1 subunit specifically in the pyramidal cells of the hippocampal CA1 region (Tsien et al., *Cell* **87**, 1317-26, 1996). These knockout mice were demonstrated to lack NMDA receptor-mediated synaptic currents and LTP in the CA1 region and exhibited impaired spatial memory, but unimpaired nonspatial learning (Tsien et al., *Cell* **87**, 1327-38, 1996). Multiple electrode recording techniques revealed that, although the CA1 pyramidal cells of the mice retained place-related activity, there was a significant decrease in the spatial specificity of individual place fields. Moreover, there was a striking deficit in the coordinated firing of pairs of neurons tuned to similar spatial locations (McHugh et al., *Cell* **87**, 1339-49, 1996). These results demonstrated that NMDA receptor-mediated synaptic plasticity is necessary for the proper representation of space in the CA1 region of the hippocampus, and strongly suggested that activity-dependent inhibition of CA1 synapses, as mediated by NMDA receptors, plays an essential role in the acquisition of spatial memories.

The foregoing results obtained with NMDA receptor knockout mice provide evidence that complete inhibition of the receptor in a specific region of the brain inhibits certain specific forms of memory. However, they do not address the role of the receptor in broader types of learning and memory, nor do they provide direction in applying such information to the improvement of learning and memory, or to treatment of diseases or disorders that detrimentally affect learning and memory.

Such neuronal degenerative disorders, which include for
example, Alzheimer's disease and stroke, are becoming
increasingly prevalent in the population as it ages. It
would be a significant advance in the field of clinical
5   neurobiology to devise a means by which such disorders
could be treated or reversed, and which further could be
applied to the improvement of learning and memory in any
individual in need of such treatment.

10   **SUMMARY OF THE INVENTION**

The present invention provides methods and
tools for improving learning or memory in a subject, for
treating learning and memory-related degenerative disease
in a patient in need of such treatment, for identifying
15   novel agents capable of regulating learning and memory
(positively or negatively), and for identifying genes
involved in biological processes related to learning and
memory.

In accordance with one aspect of the invention
20   method for improving learning or memory in a subject is
provided. The method comprises modifying NMDA receptors
in neural synapses of the subject's brain, such that the
NMDA receptor function is increased as compared with an
equivalent unmodified subject. Such an increase in NMDA
25   receptor function is measured as an increase in synaptic
plasticity and NMDA receptor activation (i.e., increase
in channel decay time or peak amplitude).

In accordance with another aspect of the
invention, a method of treating a neurodegenerative
30   disorder affecting learning or memory in a patient in
need of such treatment is provided. The method comprises
modifying NMDA receptors in neural synapses of the
patient's brain, such that the NMDA receptor function is
increased as compared with an equivalent unmodified
35   patient, the modification resulting in improved learning

or memory in the patient.

According to another aspect of the invention, a genetically altered non-human animal having enhanced learning and memory as compared with an equivalent, but

5   unaltered animal, is provided. The genetic alteration results in a modification of NMDA receptors in neural synapses of the animal's brain, such that the NMDA receptor function is increased as compared with an equivalent unaltered animal.

10   According to another aspect, the invention provides a transgenic non-human animal that expresses an NR2B transgene in its brain. In a preferred embodiment, the animal is a rodent, most preferably a mouse.

According to another aspect of the invention, a

15   method of identifying compounds that enhance learning and memory in a subject by increasing expression of NR2B genes in the subject is provided. The method comprises providing a chimeric DNA construct comprising an NR2B promoter operably linked to a reporter gene, contacting

20   the chimeric DNA construct with a test compound suspected of up-regulating the NR2B promoter, and measuring expression of the reporter gene, an increase in the expression being indicative that the test compound enhances learning and memory in the subject by increasing

25   expression of NR2B genes in the subject.

According to yet another aspect of the invention, an in vitro or in vivo assay for identifying compounds that enhance learning and memory in a subject by affecting NMDA receptor function is provided. The in

30   vitro method comprises: (a) providing a pair of cell cultures, one being transgenic and expressing an exogenous nucleic acid molecule encoding NR2B, and the other being non-transgenic for expression of an exogenous nucleic acid molecule encoding NR2B; (b) treating the

35   non-transgenic cells with a test compound suspected to

affect the expression NR2B or activity of the NMDA receptors; and (c) comparing NMDA receptor function of the treated, non-transgenic cells with NMDA receptor function of the transgenic cells and, optionally, NMDA

5   receptor function of an untreated, non-transgenic cells, a change in NMDA receptor function in the treated, non-transgenic cells that comprises the same features of NMDA receptor function exhibited in the transgenic cells being indicative that the test compound enhances learning and

10   memory in a subject by affecting NMDA receptor function.

The *in vivo* method is practiced similarly, but instead of cultured cells, transgenic and non-transgenic animals are used.

According to still another aspect of the

15   invention, another *in vitro* or *in vivo* assay for identifying compounds that enhance function of NMDA receptors in a subject is provided. The *in vitro* assay comprises: (a) providing a pair of cell cultures; (b) treating one of the cell cultures with a test compound

20   suspected of enhancing NMDA receptor function; and (c) directly or indirectly measuring a change in NMDA function in the treated cells as compared with the untreated cells, a change being indicative that the test compound affects NMDA receptor function in a subject. In

25   a preferred embodiment, the cells are transgenic NR2B-expressing cells. Use of such cells is advantageous because they are expected to exhibit more robust responses to the various test compounds.

The *in vivo* assay of this type is practice

30   similarly, except utilizing animals instead of cultured cells. In a preferred embodiment, the animals are the above-described genetically altered animals with enhanced learning and memory. Use of such animals is advantageous because they are expected to exhibit more robust

35   responses to the various test compounds.

According to yet another aspect of the
invention, a method of identifying genes and gene
products that affect NMDA receptor-mediated learning and
memory in a subject is provided. The method comprises:
5   (a) providing a pair of equivalent animals or cell
cultures, one being transgenic and expressing an
exogenous nucleic acid molecule encoding NR2B, and the
other being non-transgenic for expression of an exogenous
nucleic acid molecule encoding NR2B; (b) comparing
10   profiles of gene expression (via mRNA or protein) or
protein modification (covalent or non-covalent) in the
transgenic and non-transgenic animals or cells; (c)
isolating one or more genes or gene products whose
expression or modification is altered in the transgenic
15   animal or cells; and (d) identifying the one or more
genes or gene products.

According to still another aspect of the
invention, another method of identifying genes and gene
products that affect NMDA receptor-mediated learning and
20   memory in a subject is provided. This method comprises:
(a) providing cells containing NMDA receptors; (b)
stimulating the NMDA receptors (directly or indirectly)
in a sample of the cells; (c) comparing profiles of gene
expression (via mRNA or protein) or protein modification
25   (covalent or non-covalent) in the cell sample having
stimulated NMDA receptors with an equivalent cell sample
wherein the NMDA receptors are unstimulated; (d)
isolating one or more genes or gene products whose
expression or modification is altered in the cells having
30   stimulated NMDA receptors; and (d) identifying the one or
more genes or gene products.

Other features and advantages of the present
invention will be understood by reference to the
35   drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction and biochemical characterization of transgenic NR2B mice. Fig. 1a, The construct pJT-NR2B for production of NR2B transgenic mice. Fig. 1b, Expression of NR2B transgene mRNA in transgenic mice. Lane 1: the cortex/striatum/amygdala; Lane 2, the hippocmpus; lane 3, the brain stem and the thalamus; lane 4: the cerebellum. Fig. 1c, Synaptic NMDA receptor protein level in the hippocampus (HP) and the cortex (CTX) in both transgenic lines (Tg-1 & Tg-2) and wild-type (wt). The same membrane was used for blotting with antibodies against NR1 (120 KD), NR2A (170 KD) and NR2B (180 KD), respectively. Fig. 1d, Forebrain-specific expression of NR2B transgene revealed by *in situ* hybridization. CTX, cortex; STM: striatum; HP, hippocampus; AMG: amygdala. Fig. 1e, Normal brain morphology in transgenic mice (Nissl staining). Fig. 1f, A higher magnification of the Nissl-stained transgenic hippocampus. DG, dentate gyrus; CA1, and CA3 are marked. Fig. 1g, Golgi staining of the dendritic spines of CA1 cells from wild-type (left) and transgenic mice (right). Scale bar represents 5 μm.

Figure 2. Developmental changes in NMDA current at single synapse. Fig. 2a, Confocal image depicting a dendrite with single synapses marked by FM 1-43 (arrows). The iontophoretic electrode on the right (out of the focal plan) was brought at close proximity to the FM spot to deliver glutamate (1msec). Fig. 2b, Representative example of a current-voltage relationship of glutamate-evoked response from a single synapse from a wild type neuron. At -80 to -40 mV, non-NMDA current was exclusively observed, while at more positive potentials, both non-NMDA (peak current typically observed at 3 ms post application) and NMDA current (peak current measured 30-40 ms post-application) were recorded. The proportion

of NMDA current evoked display a tyical "J" shaped I/V
relation (black circles) while non-NMDA current varies
linearly with the membrane potential (open circles).
Fig. 2c, Representative examples of NMDA currents at +40
5   mV recorded from wt (light trace) and Tg mice (dark
trace) at day 10, 14, and 18 in vitro culture (DIV)
respectively. Insets display the same traces, normalized
and expressed as a semi-log plot to emphasize decay
portion of NMDA currents. A single exponential, which
10   provided excellent fits, was used to assess the decay
time τ and values for the representative traces are
indicated. Fig. 2d - Fig. 2f, Averaged values for peak
amplitude, decay time, and charge transfer of NMDA
currents respectively. Each point represents mean ± SEM
15   of 8 to 18 experiments per data points obtained from 18
synapses on 13 neurons from 6 wild-type mice and 31
synapses on 19 neurons derived from 9 transgenic (Tg-1 &
Tg-2 mice). (*) indicates significance between wild-type
and Tg mice (P<0.01, 2-tailed unpaired student's t test).
20   **Figure 3.** Selective enhancement of 10-100Hz-
induced potentiation in transgenic mice. Tg-1, filled
squares; Tg-2, filled triangles; wild-type (wt), open
squares. Fig. 3a, Wild-type and transgenic mouse slices
showed no significant difference in paired-pulse
25   facilitation of the EPSP at various interpulse intervals.
Fig. 3b, Transgenic slices had greater NMDA receptor-
mediated EPSP than wild-type slices. At 1.1 mV fiber
volley, the area under the $EPSP_{NMDA}$ were 124.8 ±20.6 (mV x
msec) in Tg mice and 31.1 ±5.3 (mV x msec) in controls
30   (p<0.001). Fig. 3c, A tetanic stimulation (100 Hz, 1 s)
induced significantly larger potentiation in transgenic
slices (Tg-1, 9 slices/6 mice; Tg-2, 6 slices/3 mice)
than wild-type slices (n=10 slices/8 mice); Inset:
representative records of the EPSP before and 45 min
35   after tetanus in a wt (left) and Tg (right) slice. Fig.

3d, 10 Hz stimulation for 1.5 min produced significant synaptic potentiation in transgenic slices (Tg-1, 5 slices/5 mice; Tg-2, 4 slices/3 mice) but not wild-type slices (9 slices/9 mice). Fig. 3e, LTD induced by low-frequency stimulation (1 Hz for 15 min) is similar between wild-type and transgenic slices. Fig. 3f, Summary data for synaptic plasticity at different frequencies. For comparisons, results from our previous study of CA1-specific NMDA-R1 knockout mice (open circles, dot line) was also included.

Figure 4. Enhanced novel object recognition memory in transgenic mice. Fig. 4a, Exploratory preference in the training session. The dotted line represents the performance at the chance level (50%). The amount of time in exploring the two objects was the same between transgenic and wild-type mice. Fig. 4b, Enhanced exploratory preference in transgenic mice in retention test. Figure indicates a temporary feature of the enhanced long-term memory in the transgenic mice (Tg-1=19, Tg-2=8, wt=14). Data were expressed as mean ± s.e.m. *, p,0.05; **, p<0.01, post-hoc analysis between transgenic and wild-type mice.

Figure 5. Enhancement of both contextual- and cued-fear memory in transgenic mice. Figs. 5a-5c, Contextual conditioning 1 hr, 1 day, and 10 days after training, respectively. Figs. 5d-5f, Cued-fear conditioning 1 hr, 1 day, 10 days after training, respectively. Each point represents data collected from 8-10 mice per group (wt, Tg-1, or Tg-2). The value in each column represents percentage of freezing rate and expressed as mean ± s.e.m. *, p<0.05, post hoc analysis between wild-type and transgenic mice.

Figure 6. Transgenic mice exhibit faster fear extinction. Fig. 6a, Faster fear extinction to contextual environment in transgenic mice. Either wild-

type (n = 8) or transgenic (Tg-1, n=7; Tg-2, n=8, data
were plotted together) mice were given the same single
CS/US pairing training as described in figure 5 and then
subjected to 5 extinction trials 24 hr after training.

5   Fig. 6b, Faster fear extinction to the tone in transgenic
mice. The value in each column represents percentage of
freezing rate and data were expressed as mean ± s.e.m.
*, P<0.05; **, p<0.01; ***, p<0.001, *post-hoc* analysis.

Figure 7. Enhanced performance in the hidden-
10   platform water maze task by transgenic mice. Fig. 7a,
Escape latency (mean ± s.e.m) in the water maze training
(Tg-1, n=13) or wild-type mice (n=15). Fig. 7b, Place
preference in the first transfer test conducted at the
end of 3rd training session. Transgenic mice spent more
15   time in the target quadrant than other quadrants, whereas
control mice did not show any preference for the target
quadrant at this stage. Fig. 7c, Place preference in the
second transfer test carried out at the end of the 6th
training session. Both transgenic and wild-type mice
20   exhibited strong preference for the target quadrant where
the hidden-platform was previously located. *, p<0.05,
post-hoc analysis in figure 7a, and Student' *t*-test in
figure 7b, between transgenic and controls.

25   **DETAILED DESCRIPTION OF THE INVENTION**

     I. Definitions:

          Various terms relating to the present invention
     are used throughout the specification and claims.

          A "coding sequence" or "coding region" refers
30   to a nucleic acid molecule having sequence information
     necessary to produce a gene product, when the sequence is
     expressed.

          The term "operably linked" or "operably
     inserted" means that the regulatory sequences necessary
35   for expression of the coding sequence are placed in a

nucleic acid molecule in the appropriate positions
relative to the coding sequence so as to enable
expression of the coding sequence. This same definition
is sometimes applied to the arrangement other

5   transcription control elements (e.g. enhancers) in an
expression vector.

Transcriptional and translational control
sequences are DNA regulatory sequences, such as
promoters, enhancers, polyadenylation signals,

10   terminators, and the like, that provide for the
expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or
"promoter sequence" refer generally to transcriptional
regulatory regions of a gene, which may be found at the

15   5' or 3' side of the coding region, or within the coding
region, or within introns. Typically, a promoter is a
DNA regulatory region capable of binding RNA polymerase
in a cell and initiating transcription of a downstream
(3' direction) coding sequence. The typical 5' promoter

20   sequence is bounded at its 3' terminus by the
transcription initiation site and extends upstream (5'
direction) to include the minimum number of bases or
elements necessary to initiate transcription at levels
detectable above background. Within the promoter

25   sequence is a transcription initiation site (conveniently
defined by mapping with nuclease S1), as well as protein
binding domains (consensus sequences) responsible for the
binding of RNA polymerase.

A "vector" is a replicon, such as plasmid,

30   phage, cosmid, or virus to which another nucleic acid
segment may be operably inserted so as to bring about the
replication or expression of the segment.

The term "nucleic acid construct" or "DNA
construct" is sometimes used to refer to a coding

35   sequence or sequences operably linked to appropriate

- 14 -

regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA" or "transgene". Such a nucleic acid construct may contain a

5   coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a

10 transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid

15 construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA

20 that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences

25 having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected"

30 by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA (transgene) may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming

35 DNA may be maintained on an episomal element such as a

plasmid. With respect to eukaryotic cells, a stably
transformed cell is one in which the transforming DNA has
become integrated into a chromosome so that it is
inherited by daughter cells through chromosome

5   replication. This stability is demonstrated by the
ability of the eukaryotic cell to establish cell lines or
clones comprised of a population of daughter cells
containing the transforming DNA. A "clone" is a
population of cells derived from a single cell or common

10   ancestor by mitosis. A "cell line" is a clone of a
primary cell that is capable of stable growth *in vitro*
for many generations. If germline cells are stably
transformed, the transformation may be passed from one
generation of animals arising from the germline cells, to

15   the next generation. In this instance, the transgene is
referred to as being inheritable.

         Other definitions are found in the description
set forth below.

20   **II. Description:**

         It has been widely held that mental and
cognitive attributes, such as intelligence and memory,
are the products of a variety of genetic and
environmental factors. However, the present inventor has

25   made the surprising and unexpected discovery that
alteration of a single biological event, i.e., NMDA
receptor-dependent modification of synaptic efficacy,
significantly improves associative learning and memory in
mammals.   The inventor demonstrated this unifying

30   mechanism for associative learning and memory through a
genetic manipulation of the NMDA receptor in mice. As
described in detail in the examples, the inventor has
shown that overexpression of NMDA receptor 2B (NMDAR2B,
or NR2B) in the forebrains of transgenic mice leads to

35   enhanced activation of NMDA receptors, facilitating

synaptic potentiation in response to a 10-100 Hz
stimulation. These mice exhibit enhanced capability of
both learning and memory in six behavioral tasks,
demonstrating a pivotal role of NR2B in gating the age-
5   dependent threshold for plasticity and memory formation
and, more broadly, demonstrating that enhanced activation
of NMDA receptor function leads to marked improvement in
mammalian learning and memory, through enhancement of
synaptic plasticity. Thus, the NMDA receptor has now
10   been shown to function as a "master molecular switch" for
various forms of learning and memory.

The studies with NR2B transgenic animals have
revealed fundamental biochemical changes in the NMDA
receptor that lead to the observed improvements in
15   learning and memory. These include the maintenance of a
large single synapse peak amplitude and a prolonged
channel decay, which together result in larger charge
transfer through the synaptic NMDA receptor channel, and
increased information transfer as exemplified by ion
20   influx and efflux in the NMDA receptor-containing
neurons. Therefore, overexpression of the NR2B transgene
has resulted in the prolonged opening of the NMDA
receptors for detecting coincidence and the enhanced NMDA
activation in individual synapses, thus retaining several
25   juvenile features of NMDA receptor properties.

The term "synaptic plasticity" as used herein
refers to the ability of a neuron to change its
communication efficacy based on past firing activity.
One example is the phenomenon of long-term potentiation
30   (LTP): upon a high frequency stimulation, the synaptic
communication efficacy is potentiated, and that
potentiation can last for several weeks. The term "NMDA
receptor activity" refers to parameters of the NMDA
receptor itself, such as channel decay time or peak
35   amplitude of the electrochemical signal through the

channel. The inventor has show that improvements in NMDA
receptor activity lead to enhanced synaptic plasticity.
These two features are referred to collectively herein as
"NMDA receptor function". It is the improvement or
5   enhancement of NMDA receptor function that results in
enhanced learning and memory in accordance with the
present invention.

The studies with NR2B transgenic animals
reported herein have shown that learning and memory is
10   enhanced with only a modest amount of improvement of NMDA
receptor function. For example, focusing on NMDA
receptor activity, a 15-20% increase in length of channel
decay still resulted in improved learning and memory
behaviors in the transgenic animals, as compared to non-
15   transgenic animals. Greater increases in NMDA receptor
function are preferred for practice of the invention
(e.g., 30%, 40%, 50% or more increase in channel decay
length, peak amplitude, or gating), but the threshold
appears to be relatively low.

20   The discoveries made in accordance with the
present invention can be put to practical use in a
variety of ways. One overall aspect of the invention
provides a method for improving learning or memory in a
mammal, which comprises altering an effective amount of
25   the NMDA receptors in learning or memory-associated brain
areas (e.g., hippocampus) in a manner that enhances
synaptic plasticity in neurons containing the altered
receptors.

It is believed that any means by which NMDA
30   function can be enhanced will yield the same improvements
in learning and memory as observed in the NR2B transgenic
mice. Thus, altering the ratio of NR2B to NR2A in NMDA
receptors via overexpression of NR2B is but one means by
which such a result may be achieved. Other methods to
35   enhance NMDA receptor function include, but are not

limited to: (1) use of small molecules that act directly
or indirectly to enhance NMDA receptor function; (2)
modulating expression of NMDA receptor subunits at the
transcriptional (e.g., promoter), translational (which
5   may include upregulation of upstream transcription
factors) and/or post-translational level (e.g., covalent
or non-covalent protein modification); (3) use of agents
that act inside the cell at the intracellular domains of
the NMDA receptor or one of its downstream signaling
10   molecules, thereby modulating interaction between the
receptor and its downstream targets; (4) use of agents
inside the cell to stimulate the downstream gene
expression as seen in the NMDA-activated transgenic mice
or cells described herein; and (5) enhancing the NMDA
15   receptor-mediated processes indirectly by modulating
other neuronal receptors (e.g., the AMPA receptor, GABA
receptor, serotonin receptor) or presynaptic
neurotransmitter releases, thereby affecting NMDA
receptor responses.
20   In a preferred embodiment, NR2B subunits are
targeted for modulation. As exemplified herein, NR2B may
be overexpressed in a desired region of the brain through
genetic manipulation. In one embodiment, a subject may
be stably transformed with a vector encoding NR2B
25   subunits. Stable transformation is preferably applied to
juvenile or embryonic subjects, such that the subjects
enjoy enhanced synaptic plasticity through their youth
and into adulthood. In a particularly preferred
embodiment, germline cells of embryonic subjects are
30   transformed, resulting in subjects that can pass the
transgene along to their offspring. These types of
transformation are accomplished using techniques well
know to persons skilled in the art, as described in
greater detail below.
35   Alternatively, somatic cells of subjects may be

stably or transiently transformed with a vector encoding NR2B subunits. Somatic transformation is suitable for juvenile or adult subjects, as a means to correct or reverse an existing defect or pathological condition of the brain. Such "DNA therapy", for instance would comprise targeted administration of an NR2B expression vector which, upon delivery to the target cells, would produce excess NR2B that would be incorporated into NMDA receptors. DNA therapy to transiently produce NR2B in targeted brain locations is accomplished according to methods well known in the art, as described in greater detail below.

It should be understood that the term "subject" or "patient", as used herein, refers to humans (where appropriate) and also to non-human animals. The methods of the invention may be applied to any organism having a central nervous system that contains NMDA receptors. Mammals are preferred, and humans are particularly preferred, especially wherein the methods are used to treat or alleviate a neurological pathological condition or disease.

In preferred embodiments, NMDA receptors of the forebrain are targeted for enhancement, including the cortex, striatum, hippocampal structures, hippocampal formation, amygdala, and limbic system. Other learning-associated brain areas also may be targeted, including the cerebellum and thalamic region, nucleus accumbens and basal ganglion. NMDA receptor enhancement can be used in any area of the nervous system in which the receptors are located, to achieve a variety of desirable results. Such therapy is expected to be useful for the treatment of various learning and memory disorders, including schizophrenia, Alzheimer's disease and other age-related learning or memory impairment and amnesia of all types, including memory deficit resulting from drug or alcohol

abuse, as well as for the restoration of capacity to individuals who have suffered brain damaged due to, e.g., head trauma, stroke or ischemia (e.g., by drowning).

5   It should also be appreciated that, since enhancement of NMDA receptor function improves learning and memory, then impairment of NMDA receptor function should impair learning and memory. Such impairment also may have therapeutic utility, and the present invention is also drawn to compositions and methods for impairing

10 NMDA receptor function. This therapy is expected to be useful in instances where cognitive suppression is desired, such as in the management of chronic pain (a form of plasticity or memory) or for purposes of creating selective amnesia with respect to a traumatic event.

15 The present invention also provides genetically modified non-human animals, and cells of those animals, which have been altered in NMDA receptor function so as to display enhanced synaptic plasticity as compared with an equivalent, but unaltered animal. The genetic

20 modification made to such animals can be one of many types, including introduction of an exogenous gene (transgenic animals as described in greater detail below), or up-regulation or down-regulation of an endogenous gene, by natural or artificial mutagenesis or

25 by transgenic manipulation.

In a preferred embodiment, NR2B transgenic animals are provided, which are expected to be useful for a variety of purposes, as discussed in greater detail below. As exemplified by the NR2B transgenic mice

30 described herein, these animals exhibit enhanced capabilities relating to acquiring new information (i.e., learning) and storing existing information (i.e., memory).

The term "animal" is used herein to include all

35 vertebrate animals, except humans. It also includes an

individual animal in all stages of development, including
embryonic and fetal stages. Examples of animals
preferred for use in the present invention include, but
are not limited to, rodents, most preferably mice and
5   rats, as well as cats, dogs, dolphins and primates.

A "transgenic animal" is any animal containing
one or more cells bearing genetic information altered or
received, directly or indirectly, by deliberate genetic
manipulation at the subcellular level, such as by
10   targeted recombination or microinjection or infection
with recombinant virus. The term "transgenic animal" is
not meant to encompass classical cross-breeding or *in
vitro* fertilization, but rather is meant to encompass
animals in which one or more cells are altered by or
15   receive a recombinant DNA molecule, i.e., a "transgene".
The term "transgene", as used herein, refers to any
exogenous gene sequence which is introduced into both the
somatic and germ cells or only some of the somatic cells
of a mammal. This molecule may be specifically targeted
20   to defined genetic locus, or be randomly integrated
within a chromosome, or it may be extrachromosomally
replicating DNA. The term "germline transgenic animal"
refers to a transgenic animal in which the transgene was
introduced into a germline cell, thereby conferring the
25   ability to transfer the transgene to offspring. If such
offspring in fact possess the transgene then they, too,
are transgenic animals.

The transgene of the present invention includes
without limitation, the entire coding region of an NR2B
30   gene, or its complementary DNA (cDNA), or chimeric genes
containing part or all of a NR2B coding region, whose
expression in the forebrain is driven by a tissue specific
promoter. It is preferable, but not essential, that the
NR2B coding sequence used in the transgene be of the same
35   species origin as the transgenic animal to be created.

Nucleic acid sequences encoding NR2B have been reported for several species. Examples include mouse (GenBank Accession No. U60210) and human (GenBank Accession No. NM 000834).

5 The promoter is comprised of cis-acting DNA sequences capable of directing the transcription of a gene in the appropriate tissue environment and, in some cases, in response to physiological regulators. The promoter preferred for use in the present invention is

10 derived from the αCaMKII gene, whose activity has been demonstrated to be restricted to the forebrain region (Mayford et al., Cell **81**, 891-904, 1995). Other promoters are also known to direct the expression of exogenous genes to specific cell-types in the brain.

15 Promoters useful for stem cell transformation, wherein tissue specificity is needed, include any promoter whose endogenous genes are expressed in the target cell of interest; e.g., the pkcγ promoter, the telencephalin promoter, the neuronal enolase promoter and the prp

20 promoter. For somatic transformation, tissue specific promoters may or may not be needed. Thus, constitutive promoters, such as the CMV promoter or the β-actin promoter should prove useful for somatic transformation.

Methods to obtain transgenic, non-human mammals

25 are known in the art. For general discussions, see, e.g., Joyner, "Gene Targeting," IRL Press, Oxford, 1993; Hogan et al. (Eds.), "Manipulating the Mouse Embryo - A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1994; and Wasserman &

30 DePamphilis, "A Guide to Techniques in Mouse Development," Academic Press, San Diego CA, 1993. One method for introducing exogenous DNA into the germline is by microinjection of the gene construct into the pronucleus of an early stage embryo (e.g., before the

35 four-cell stage) (Wagner et al., *Proc. Natl. Acad. Sci.*

USA **78**, 5016, 1981; Brinster et al., *Proc. Natl. Acad. Sci.* USA **82**, 4438, 1985). The detailed procedure to produce NR2B transgenic mice by this method has been described (Tsien et al., *Cell* **87**, 1317-26, 1996).

5   Another method for producing germline transgenic mammals utilizes embryonic stem cells. The DNA construct may be introduced into embryonic stem cells by homologous recombination (Thomas et al., *Cell* **51**, 503, 1987; Capecchi, *Science* **244**, 1288, 1989; Joyner, et al.,

10   *Nature* **338**, 153, 1989) in a transcriptionally active region of the genome. A suitable construct may also be introduced into the embryonic stem cells by DNA-mediated transfection, such as electroporation (Ausubel, et al., Current Protocols in Molecular Biology, John Wiley &

15   Sons, 1999). Detailed procedures for culturing embryonic stem cells and methods of making transgenic mammals from embryonic stem cells may be found in Teratocarcinomas and Embryonic Stem Cells, A practical Approach, ed. E. J. Robertson (IRL Press, 1987).

20   Other methods for producing germline transgenic animals are being developed currently. For instance, instead of eggs being the recipients of exogenous DNA, sperm are now being genetically manipulated or targeted by mutagenic agents.

25   In any of the foregoing methods of germline transformation, the construct may be introduced as a linear construct, as a circular plasmid, or as a viral vector which may be incorporated and inherited as a transgene integrated into the host genome. The transgene

30   may also be constructed so as to permit it to be inherited as an extrachromosomal plasmid. The term "plasmid" generally refers to a DNA molecule that can replicate autonomously in a host cell.

          Transgenic animals also may be obtained by

infection of neurons either *in vivo, ex vivo,* or *in vitro*
with a recombinant viral vector carrying an NR2B gene.
Suitable viral vectors include retroviral vectors,
adenoviral vectors and Herpes simplex viral vectors, to

5   name a few. The selection and use of such vectors is
well known in the art.

The present invention also provides a variety
of assays and other methods, which utilize the inventors'
discovery of the profound effect of NMDA receptor

10   function enhancement on synaptic plasticity, learning and
memory.

One useful assay is an *in vitro* assay for
identifying compounds that enhance learning and memory by
increasing expression of NR2B genes. This assay involves

15   the following basic steps: (1) provide a chimeric DNA
construct comprising an NR2B promoter operably linked to
a reporter gene; (2) contact the chimeric DNA construct
with a test compound suspected of up-regulating the NR2B
promoter, and (3) measure expression of the reporter

20   gene. An increase in the expression of the reporter gene
indicates that the test compound will enhance learning
and memory by increasing expression of NR2B genes.

The NR2B transgenic animals of the invention
may be used for several *in vivo* assays. For instance,

25   they may be used as follows for identifying compounds
that enhance learning and memory by affecting expression
of NR2B or activity of NMDA receptors: (1) provide a
pair of equivalent animals, one being NR2B transgenic,
and the other being non-transgenic; (2) treat the non-

30   transgenic animal with a test compound suspected to
affect the expression or activity of the NR2B; (3)
compare (biochemically or using behavioral tests)
learning and memory of the treated, non-transgenic mammal
with learning and memory of the transgenic mammal (and,

35   optionally, learning and memory of an untreated, non-

transgenic mammal). Any change in learning and memory in
the treated, non-transgenic mammal that comprises the
same features of learning and memory exhibited in the
transgenic mammal would be an indicator that the test
5   compound enhances learning and memory in the mammal by
affecting expression of NR2B or activity of NMDA
receptors.

Another *in vivo* assay, useful for identifying
compounds that affect activation of NMDA receptors in a
10 mammal, comprises the following steps: (1) provide a pair
of animals; (2) treat one with a test compound suspected
of affecting NMDA receptor function; and (3) directly or
indirectly measure a change in activity of the treated
animal as compared with the untreated animal, a change
15 being indicative that the test compound NMDA receptor
function in the animal. This assay can be modified by
the use of a pair of NR2B transgenic animals, or by
comparing an NR2B transgenic animal with a non-transgenic
animal and/or with a transgenic "knockout" animal, whose
20 NMDA receptors are dysfunctional or non-functional (e.g.,
as described by Tsien et al., 1996, *supra*).

The NR2B transgenic animals of the invention
may also be used in method of identifying genes and gene
products that affect NMDA receptor-mediated learning and
25 memory. Such an assay has the following steps (1)
provide a pair of equivalent animals, one being NR2B
transgenic, and the other being non-transgenic; (2)
compare profiles of gene expression (via mRNA or protein
production) or post-translational modification) in the
30 transgenic and non-transgenic animals; (3) isolate one or
more genes or gene products whose expression is altered
or which is modified in the transgenic animal; and (4)
identify the one or more genes or gene products. This
assay also can be modified by comparing the NMDA
35 transgenic and non-transgenic animals with a transgenic

"knockout" animal. Another adaptation of this type of
assay is to subject non-transgenic animals to chemical or
electrical neuronal stimulation to enhance NMDA function,
then to monitor the gene expression profile of the
5   treated animals and compare it to the expression profile
observed in the NR2B transgenic animals.

It will be appreciated that assays similar to
the *in vivo* assays discussed above can be developed
easily in cultured cells. For instance, cultured
10   neuronal or non-neuronal cells may be transformed with a
DNA construct for expression of NR2B, optionally together
with NR1 subunit or other NR2 subunits (NR2A, NR2C,
NR2D), and those cells used for various biochemical and
physiological assays to assess the changes resulting from
15   the presence of the transgene. In another embodiment,
cells or tissue slices from NR2B transgenic animals may
be utilized for a similar purpose.

In a simple, but preferred embodiment, non-
transgenic cultured cells of a selected type are used in
20   an assay to screen for compounds that can enhance
learning and memory by improving NMDA receptor function.
The cells are exposed to a test compound and NMDA
receptor activity is measured by standard biochemical or
electrophysiological means. If NMDA receptor function is
25   improved in comparison to untreated cells, the test
compound is suitable for further analysis as a
pharmaceutical compound for enhancing learning and
memory.

The discoveries made in accordance with the
30   present invention also suggest other methods for
identifying genes and gene products that affect NMDA
receptor-mediated learning and memory in a subject. For
instance, identifying proteins (and genes encoding them)
that physically interact with the receptor or with genes
35   encoding the receptor can yield useful information

relating to NMDA receptor signaling pathways in the cell. Such interactions may be identified by several methods known in the art, including (1) the yeast two-hybrid system, (2) phage display and (3) immunoprecipitation.

5   The inventor's discovery that the NMDA receptor serves as a "master switch" in learning and memory also points to diagnostic assays based on polymorphisms or mutations in the genes encoding the receptor. Such polymorphisms or mutations, which could occur in the

10   coding region or the non-coding region, are identified in subjects outside the norm (above or below) with respect to a selected form of learning or memory, and these polymorphisms are correlated with either the enhancement or the impairment of the selected form of learning or

15   memory. Once identified, these polymorphisms can be used as a genetic screen to predict Intelligence Quotient or other measurements of learning and memory capability, or predisposition to learning or memory disorders.

          The foregoing assays and methods are only a few

20   of many that can be developed as a result of the discoveries made in accordance with the present invention. Persons of skill in the art will find numerous additional ways to use this information, and the biological tools and animal model systems described

25   herein.

          The following examples are set forth to illustrate embodiments of the invention. They are not intended to limit the scope of the invention in any way.

30   The examples describe experiments performed to test the notion that the NR2B subunit is crucial for implementing Hebb's rule and gating synaptic plasticity and memory. To accomplish this, the NR2B subunit was overexpressed postnatally in mouse forebrains. Of seven lines

35   produced, the examples describe results from two

independent lines (Tg-1 and Tg-2), which have been
systematically analyzed and have been found to have
similar expression patterns, levels, and nearly identical
electrophysiological and behavioral phenotypes.

5

Example 1
Production and Basic Characterization
of NR2B Transgenic Mice

10   The linearized NR2B transgene expression vector
pJT-NR2B containing the CaMKII promoter and the NR2B
transgene was obtained by digestion of pJT-NR2B with Sal
and purified away from plasmid sequence. The transgenic
founders were produced by pronuclear injection of the
15   linearized DNA into C57B/6 inbred zygotes as described
(Tsien et al., *Cell* **87**, 1317-26 1996). The inbred
founders were crossed into either C57B/6 or CBF1 to
produce F1 generation. The F2 offsprings derived from
intercross between C57B/6 and CBF1 were used for various
20   analyses. We found that F2 wild-type mice on this hybrid
background consistently showed excellent learning
behaviors, which are critical to any comparative
behavioral studies. This mating strategy, therefore,
sets a much higher standard for our behavioral
25   enhancement experiments.
          The genotypes of all offspring were analyzed by
preparing tail DNAs. The 5' and 3' primers for detecting
NR2B transgene SV40 polyA sequence (505 bp) were 5'-
AGAGGATCTTTGTGAAGGAAC-3' (SEQ ID NO:1) and 5'-
30   AAGTAAAACCTCTACAAATG-3' (SEQ ID NO:2), respectively.
Mouse tail DNAs (about 1μg) were amplified 30 cycles (1
min, 94°C; 45 sec, 55 °C; 1 min, 72 °C) on a thermal
cycler. For detecting transgene mRNA, a SV40 poly(A)
tail fragment was used for Northern blot. For Western
35   blot, the antibodies against NR1, NR2A, and NR2B were
purchased from Upstate Biotechnology. Synaptic membrane

proteins were prepared from the mouse forebrain. The
sample were resolved on 7.5% SDS-polyacrylamide gel
followed by immunoblotting with the above antibodies
respectively, detected by peroxidase-labeled secondary
5   antibodies and the ECL detection system (NEN Life Science
products). For *in situ* hybridization, mouse brains were
dissected and rapidly frozen. Crytostat sections (20 μm)
were prepared and postfixed for 5 min in 4% PFA in PBS
buffer (pH7.5). The slices were hybridized to the $[\alpha^{35}S]$
10   oligonucleotide probe (5'-GCAGGATCCGCTTG
GGCTGCAGTTGGACCT-3'; SEQ ID NO:3), which hybridizes to
sequences present in the 5' untranslated artificial
intron region unique to the transgene. The detailed
procedures were the same as previously described
15   (Mayfield et al., *Cell* **81**, 891-904 1995).

RESULTS

         The transgenic animals, named *Doogie*, appeared
to be normal in their growth, body weights, and to mate
20   normally. Their open field behaviors were also
indistinguishable from those of wild-type littermates.
In addition, we did not observe any signs of seizure or
convulsion in transgenic animals. Northern blot analysis
showed that the NR2B transgene expression was enriched in
25   the cortex and the hippocampus, with little expression in
the thalamus, the brainstem and the cerebellum (Fig. 1b).
Western blot analysis revealed approximately a 1-fold
increase in the level of cortical and hippocampal NR2B
protein in transgenic mice (Fig. 1c). We have also found
30   that there is a small increase in NR1 protein level but
no change in NR2A level in these regions (Fig. 1c). This
indicates that both the ratio of NR2B over NR2A in the
receptor complex and the total number of the NMDA
receptors may be increased.
35   We investigated the transgene's anatomical

distribution using *in situ* hybridization and found that the transgene was highly enriched in the cortex, striatum, hippocampus, and amygdala (Fig. 1d). At light microscopic level, we found no gross struccutral
5   abnormalities in these transgenic animals (Fig. 1e, f). In addition, the shapes and architecture of dendritic spines of the hippocampus and the cortex are also normal (Fig. 1g).

10   **Example 2**
                    Electrophysiological Analyses of
                         NR2B Transgenic Mice

     METHODS:
15   **Hippocampal cell culture and recording.**
     Primary cultures of hippocampal neurons were prepared from individual neonatal mice (P1). Whole cell patch recordings were carried out as described elsewhere (Liu et al., *Neuron* **22**, 395-409, 1999). The composition of
20   the FM 1-43 (Molecular probe, Eugene, Or.) staining solution was KCl 90 mM, NaCl 39 mM, Glucose 30 mM, HEPES 25 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, and FM 1-43 0.01 (adjusted to pH 7.4 with NaOH). Recordings were made with 200B integrating patch clamp amplifier (Axon Instruments) with
25   a 1 kHz (8 pole Bessel) low-pass filter. Data were digitized at 10 kHz using a Digidata 1200B A/D converter (Axon Instruments). Glutamate currents were evoked by iontophoresis as described (Liu et al., 1999, *supra*). Briefly, following a one minute incubation in the FM1-43
30   solution, neurons, continuously perfused with tyrode, were visualized under confocal microscope (Olympus Fluoview) using a 40x planachromat water immersion objective. Following placement of the iontophoresis electrode, brief (1ms) glutamate pulses of varied
35   amplitudes were delivered to an isolated FM-labeled presynaptic bouton.

Hippocampal slice recording. Transverse slices of the hippocampus from transgenic and wild-type littermates (4-6 month old) were rapidly prepared and maintained in an interface chamber at 28°C, where they

5   were subfused with ACSF consisting of 124 mM NaCl, 4.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.0 mM Na_2HPO_4, and 10 mM glucose, bubbled with 95% O_2 and 5% CO_2. Slices were kept in the recording chamber for at least two hours before the experiments. A bipolar tungsten

10 stimulating electrode was placed in the stratum radiatum in the CA1 region and extracellular field potentials were recorded using a glass microelectrode (3-12 MΩ, filled with ACSF) also in the stratum radiatum. Stimulus intensity was adjusted to produce a response of

15 approximately 1 mV amplitude, with an initial slope of approximately -0.5 mV/msec. Test responses were elicited at 0.02 Hz. Homosynaptic LTD was induced by prolonged low frequency stimulation (1 Hz for 15 min). LTP was induced by tetanic stimulation (100 Hz for 1 sec).

20 Paired-pulse facilitation (PPF) of the response at various interpulse intervals (25-400 msec) was also measured. In depotentiation experiments, the stimulus to produce LTP was 100 Hz for 1 sec, delivered twice with an interval of 20 sec. This was followed by a low frequency

25 stimulus of 5 Hz for 3 min to produce depotentiation. Data are presented as mean ± s.e.m.. One-way analysis of variance (with Duncan's multiple range test for post hoc comparison) and Student's *t*-test were used for statistical analysis.

30

RESULTS

To evaluate the elementary properties of the NMDA receptors in single synapses, we employed a novel single bouton recording technique (Liu et al., 1999,

35 *supra*). Using FM 1-43 as a label of functional synaptic

- 32 -

sites in cultured hippocampal neurons, we positioned the tip of an iontophoretic electrode containing 150 mM glutamate adjacent to a relatively isolated synapse (Fig. 2a), and applied glutamate to determine the functional properties of glutamate receptors located in that particular synapse. Glutamate-evoked responses consisted of either AMPA current alone or both AMPA and NMDA currents depending upon the cell potential. The NMDA component was identified by its "J" shape of current-voltage relationship and long decay time (Fig. 2b). Thus, NMDA currents were isolated by clamping the cells to +40 mV to remove voltage-dependent Mg^{2+} block. While initial experiments were carried out in the presence of 5 µM DNQX to the bath solution to block AMPA receptors, we subsequently omitted the antagonist as NMDA current could clearly be isolated from the AMPA based on their respective time courses, and all the experiments were conducted in blind fashion.

We first determined the dose-response relation of the synaptic NMDA receptors to glutamate (an index of glutamate affinity for the NMDA receptor) and its voltage-dependent Mg^{2+} block, and found no difference between the two groups of mice. Since the recombinant NR2B subunit determines the decay phase of NMDA currents *in vitro*, we next measured NMDA channel decay time from currents evoked by a saturating dose of glutamate (100 nA of iontophoretic current, as determined from the dose-response relationship) in both transgenic and wild-type neurons. While we found no difference in decay time at day 10 or 14 in vitro (DIV), the decay time of the NMDA currents from transgenic neurons at day 18 DIV was 1.8 fold longer than those of wild-type neurons (depicted in the insets of Fig 2c and summarized in Fig. 2e, p<0.005). In addition, overexpression of the NR2B transgene resulted in the retention of the juvenile-like, single

synapse peak NMDA current amplitude over time in culture.
This is in contrast to that of wild-type neurons which
decreased significantly by 18 DIV [Fig 2d, significance
between NR2B (n=18) and wild-type (n=8), p<0.01,
5   significance between 14 (n=8) and 18 DIV (n=8) for wild-
type p<0.01]. This suggests that the total number of the
NR2B-containing NMDA receptors per single synapse is also
higher than that of wild-type animals at this stage.
These age-dependent changes in channel decay time and
10   peak amplitude are consistent with *in vivo* observation
that the NR2B transgene mRNA was detectable but low at
P14, and gradually increased to a steady level
approximately a week later.

The prolonged decay time and the maintenance of
15   large single synapse peak amplitude should result in
larger charge transfer through the synaptic NMDA receptor
channel. As such, we calculated the total amount of
charge transfer associated with the activation of the
NMDA receptors at a single synapse by integrating the
20   area between the time of glutamate application and 400 ms
later. Results clearly indicate that, at 18 DIV, the
total charge transfer through single synapse NMDA
receptors was about 4-fold larger in transgenic mice than
that of controls [2.5 ± 0.7 pC (n = 8) in wild-type
25   versus 9.8 ± 1.7 pC (n = 18) in NR2B mice p<0.001] (Fig.
2f). Therefore, overexpression of the NR2B transgene has
resulted in the prolonged opening of the NMDA receptors
for detecting coincidence and the enhanced NMDA
activation in individual synapses, thus retaining several
30   juvenile features of NMDA receptor properties.

Since blockade of the NMDA receptors prevents
the induction of major forms of plasticity such as LTP
and LTD2, we examined whether the increased time window
of coincidence detection via the NMDA receptors leads to
35   the enhancement of synaptic plasticity in the CA1 region

- 34 -

of the hippocampus. Using the hippocampal slices
prepared from the 4-6 months-old animals (in blind
fashion), we first measured the NMDA-mediated field EPSPs
in both adult wild-type and transgenic mice in the

5   Schaffer collateral CA1 path. The NMDA receptor-mediated
EPSPs were isolated in the presence of 10 µM CNQX and 0.1
mM Mg^{2+}. We found that the NMDA receptor-mediated field
EPSPs in transgenic mice were significantly greater than
those in wild-type mice, suggesting that the

10   overexpression of NR2B has resulted in the enhancement of
NMDA receptor-mediated field responses (Fig. 3b). In
addition, we confirmed that the observed synaptic
responses were NMDA receptor-dependent because it was
sensitive to NMDA receptor antagonist, 100 µM AP-5 (n=3).

15   We then studied AMPA receptor-mediated
responses and found no difference in AMPA-mediated field
EPSPs between transgenic (n = 62 slices/14 mice) and
wild-type littermates (n = 50 slices/16 mice, data not
shown). Furthermore, paired-pulse facilitation, which

20   gives an indication of presynaptic function, was similar
between transgenic mice (n = 11 slices/7 mice) and wild-
type hippocampal slices (n = 7 slices/5 mice) (Fig. 3a).
These results suggest that both presynaptic function and
postsynaptic AMPA receptors are normal in transgenic

25   animals.

         To provide overall assessment of bidirectional
synaptic plasticity in 1-100 Hz range (Dudek et al., *J.
Neurosci.* **13**, 2910-2918, 1993), we conducted a series of
LTP/LTD experiements in the Schaffer collateral CA1

30   pathway in blind fashion. We found that a single tetanic
stimulation (100 Hz, 1 s) typically evoked smaller but
reliable potentiation in 4 to 6 month-old control slices
in comparison to that in younger adult slices. However,
the same stimulus evoked significantly larger

35   potentiation in transgenic slices (Fig. 3c; transgenic, n

= 9 slices/6 mice; wild-type mice, n = 10 slices/8 mice).
The enhancement of potentiation was not due to changes in
inhibitory GABAergic mechanisms since it was similarly
observed in the presence of 100 µM picrotoxin

5   (transgenic, n = 4 slices/3 mice, mean 241.1 ± 48.2%;
wild-type, n = 5 slices/5 mice, mean 140.3 ± 19.1%;
P<0.05 compared to transgenic mice). Moreover, the
enhanced LTP was completely blocked by the application of
NMDA receptor antagonist, AP-5 (100 µM), in the bath. In

10   addition, we noted that in contrast to the normal fast
decay of fEPSP in control slices during the first 10
minutes after tetanic stimulation, there was no decay at
all, and it remained maximally potentiated, a feature
resembled with the juvenile LTP observed in postnatal day

15   15 animals (Harris et al., *J. Physiol.* (Lond) **346**, 27-48,
1984).

        The enhanced long-term potentiation in the
transgenic slices was further observed when we applied
prolonged, repetitive stimulation (10 Hz) to Schaffer-CA1

20   path. We found that while the 10 Hz stimulation for 1.5
min (900 pulses) did not induce reliable synaptic
potentiation in control animals (n = 9 slices/9 mice), it
was fully capable of evoking robust synaptic potentiation
in transgenic slices (n = 5 slices/5 mice) (Fig 3d).

25   However, repetitive stimulation delivered at 5 Hz for 3
min with the same amount of pulses (n = 900) did not
produce significant synaptic potentiation in both groups
of mice (transgenic, n = 5 slices/5 mice; mean 96.8 ±
20.3%; wild-type, n = 5 slices/5mice, mean 85.0 ± 16.4%).

30   We then investigated long-lasting synaptic
depression-induced by low frequency (Bear et al., 1994,
supra; Dudek et al., 1993, *supra*). We found that 1 Hz
stimulation produced a similar LTD in both control animals
(n = 6 slices/6 mice, 76.0 ± 9.3%) and transgenic mice (n

35   = 8 slices/7 mice, 76.8 ± 13.6%) (Fig. 3e). In addition,

we also examined synaptic depression using another
protocol in which low-frequency stimulation (5 Hz, 3 min)
was applied 5 min after strong tetanic stimulation (100
Hz, 2 x 1 s) (Stubli & Chun, *J. Neurosci.* **16**, 853-60,

5   1996). Again, similar depression or depotentiation was
induced in slices of transgenic mice (n = 4 slices/4 mice;
129.3 ± 12.9%) and wild-type mice (n = 6 slices/6 mice,
111.1 ± 14.8%). As summarized in Fig. 3f, these results
show that the enhanced NMDA receptor activation in

10   transgenic mice results in selective enhancement of long-
lasting synaptic potentiation evoked by the 10-100 Hz
frequency stimulation.

What are the effects of the selective
enhancement of 10-100 Hz LTP responses on learning and

15   memory? Since forebrain neurons often fire in this range
during behavioral experience (e.g. hippocampal neurons
fire in 4-12 Hz range, known as the θ rhythm, whereas
various cortical neurons oscillate in 20-60 Hz, known as
the γ frequency), it is likely that selective enhancement

20   of potentiation above 10 Hz in the transgenic mice could
be particularly meaningful. We previously demonstrated
that the conditional knockout of the NMDA receptor 1
subunit in the CA1 region leads to complete loss of
synaptic changes in the 1-100 Hz frequency range (Tsien

25   et al., *Cell* **87**, 1327-38, 1996) (Fig. 3f) and impairs
performance in spatial water maze. This indicates that
the normal frequency-response in this range is essential
for learning and memory. A systematic downward shift
(producing LTD) in this specific range can cause learning

30   impairment (Mayfield et al., 1995, *supra*), whereas an
upward shift (producing LTP) in all frequencies (1-100
Hz) is also deleterious to spatial learning (Migaud et
al., *Nature* **396**, 433-439, 1998). Therefore, these
observations collectively point to the importance of

35   normal 1-100 Hz responses for learning and memory.

Example 3
Behavioral Analyses of
NR2B Transgenic Mice

5   **METHODS**

Behavioral tests. Adult transgenic and wild-
type mice (3-6 months-old littermates) were used. Mice
were maintained under the standard condition (23±1 °C,
50±5 % humidity) in the animal facility. All experiments
10 were conducted in a soundproofed and specialized behavior
room. All experimenters were blind to the genotype of
the individual animal.

Novel Object Recognition Task. The apparatus,
an open-field box (20 x 20 x10 high inches), was
15 constructed from plywood and painted black with non-
toxicity paint. Before training, mice were individually
habituated by allowing them to explore the open-field box
for 5 min per session for three sessions per day and for
3 days. During training session, two novel objects were
20 placed into the open-field 14 inches away from each other
(symmetrically) and then the individual animal was
allowed to explore for 5 min. Exploring to object was
considered when the head of animal was facing the object
within 1 inch away from the object or any part of the
25 body except the tail was touching the object. The time
spent to explore each object was recorded. The animals
were returned to their home cages immediately after
training. During retention test, the animals were placed
back into the same open-field box again after certain
30 intervals, and allowed to explore freely for 5 min. Now,
one of the familiar objects used during training was
replaced by a novel object. All objects were balanced in
term of physical complexity and were emotionally neutral.
Moreover, the open-field and objects were thoroughly
35 cleaned by 70% alcohol after each session to avoid
possible instinctive odorant cues. A preference index, a

ratio of the amount of time spent exploring any one of
the two items (training session) or the novel object
(retention session) over the total time spent exploring
both objects, was used to measure recognition memory.

5   Two-way ANOVA (group X retention time) and *post hoc*
Dunnett's test were used to determine genotype effects on
the behavioral responses.

    <u>Fear Conditioning Task</u>. The fear conditioning
shock chamber and the TruScan multi-parameter activity

10   monitors were used (Coulbourn Instrument). Briefly, it
consists of a shock chamber (10 X 10 X 15 inches high)
with a 24-bar inescapable shock grid floor, a multi-tone
producer and speaker, an electrical-shock producer, a
photobeam-scanner, and a workstation. The walls of the

15   chamber are transparent, thus freezing responses of mice
in the chamber could be observed by experimenters from
the peep window on the curtain. Before training, animals
were individually habituated to the chamber a day before
the experiment for 5 min per session and three sessions

20   total. Conditioned stimulus (CS) used was an 85dB sound
at 2800Hz, and unconditioned stimulus (US) was a
continuous scrambled foot shock at 0.75 mA. During the
training, mice were put individually into the chamber and
allowed to explore the environment freely for 3 min, and

25   then were exposed to the CS for 30 sec. At the last 2
sec of the CS, the US was delivered for 2 sec. After the
CS/US pairing, the mice were allowed to stay in the
chamber for another 30 sec and then returned to their
homecages immediately. Throughout these procedures,

30   freezing responses were recorded simultaneously
experimenters using a 5-sec interval time-sampling method
as well as the photobeam-scanner system. Freezing was
judged as complete immobile of the body except for the
respiratory movements. Freezing response during the 30

35   sec after shock was recorded as immediate freezing.

During the retention test, each mouse was placed into the
shock chamber and freezing response was recorded for 3
min in this context (contextual conditioning).
Subsequently, the mice were put into a novel chamber

5   (triangular box with a smooth flat floor and yellow-black
walls) and monitored for 3 min before the onset of the
tone (pre-CS).   Immediately after that, a tone identical
to that in the training session was delivered for 3 min
and freezing responses were recorded (cued conditioning).

10   Two-way ANOVA (group X retention time) and *post hoc*
Dunnett's test were used to determine genotype effects on
the behavioral responses.

Fear Extinction Experiment.   Another groups of
transgenic and control mice were used for this

15   experiment.   Twenty-four hours after training as
described above, the mice were given a first extinction
trial.   Each extinction trial consisted of contextual and
cued extinction.   The mice were first put individually
into the shock chamber and observed for 3 min in the

20   absence of electric shock (US) for the measurement
contextual extinction.   Then, the mice were transferred
into a novel box for the measurement of cued fear
extinction.   The freezing responses were observed for 3
min in the absence of the tone (pre-CS) and subsequently

25   with the identical tone used in the training session for
another 3 min.   Following this, the 4 same extinction
trials were given at an interval of 2 hr and freezing
responses were recorded throughout the texts.   Two-way
ANOVA (group X extinction trial) and *post hoc* Dunnett's

30   test were used to determine genotype effects on the
freezing responses.

Water Maze Task.   The apparatus for water-maze
is consisted of a circle pool (1.2 m in diameter).   The
procedure was essentially the same as described

35   previously (Tsien et al., 1996, *supra*).   The training

protocol consisted of 6 sessions (4 trials/session/day).
The movement of mice was tracked by a videocamera, and
the escape latency to the platform was recorded. One-way
ANOVA and *post hoc* Dunnett's test were used to determine
genotype effects on the escape latency. In addition, two
transfer tests were performed. The first one was carried
out at the end of third session and the second one at the
end of the last session. During the transfer test, the
platform was removed and the mice were allowed to swim in
the pool for 60 sec. The time spent in each quadrant was
recorded. Student's *t*-test was used to determine
genotype effect on the spatial preference.

RESULTS

To define whether the selective enhancement of
10-100 Hz responses represents an optimal plasticity
curve, we thus conducted various learning tasks with
clear relationships to the forebrain regions, and all the
behavioral experiments were performed in a blind fashion
with respect to the genotype of the individual mouse.

We first used the novel object recognition task
to measure visual recognition memory, which is
evolutionarily conserved in various species ranging from
humans to rodents and requires the hippocampus (Reed &
Squire, *Behav. Neurosci.* **111**, 667-75, 1997; Myhrer,
Behav. Neurosci. **102**, 356-62, 1988; Mumby et al., *Behav.
Neurosci.* **110**, 266-81, 1996). To increase the difficulty
of this task, we used a 5-min training protocol (see
methods). In training session, there was no significant
difference in the amount of time spent on exploring the
two objects as shown by the exploratory preference (Fig.
4a), indicating both types of mice have the same levels
of motivation and curiosity for exploring these two
objects. During retention tests, one of the familiar
object used in the training session was replaced with a

third novel object, animals were allowed to explore for 5
min.   Both transgenic and wild-type mice exhibited the
similar levels of preference toward the novel object at
the 1hr retention test (Fig. 4b).   This suggests that all
5   groups possessed the same capability to retain the memory
of the old object for 1 hour.   However, when retention
tests were conducted either 1 day or 3 days later (Fig.
4b), both transgenic lines exhibited much stronger
preference for the novel object than wild-type mice
10     $[F(2,38) = 5.448, p < 0.01]$, indicating that transgenic
mice have better long-term memory.   A *post hoc* analysis
by using Dunnett's test reveals a significant difference
between wild-type and either transgenic line at 1-day (p
< 0.01) or 3-day retention test (p < 0.01), but not
15   between the two transgenic lines.   The observed
enhancement of long-term memory is, thus, independent of
transgene integration locus.   It should be noted that,
however, by 1 week after training, the preference in
transgenic mice also returned to the basal level.
20   We then assessed two forms of associative
emotional memories in these mice: contextual and cued
fear conditionings.   Animals learn to fear a neutral
conditioned stimulus (CS; such as a tone) which was
previously paired with aversive unconditional stimulus
25   (US; such as foot shock) or a context in which the
animals were conditioned by the pairing of CS and US.   It
has been shown that contextual fear conditioning is
hippocampal dependent whereas cued fear conditionings is
hippocampal independent (Phillips & LeDoux, *Behav.*
30   *Neurosci.* **106**, 274-85, 1992).   These two types of fear
conditioning require the activation of the NMDA receptors
(Kim et al., *Behav. Neurosci.* **106**, 591-6, 1992; Davis et
al., In: The psychology of learning and memory (Bower GH.
Ed). New York: Academic Press (1987).
35

Both contextual and cued conditioning were measured at 1 hr, 1day, and 10 days after training using separate batches of animals. We first analyzed contextual fear memory, as shown in Fig 5a-c, transgenic

5   mice consistently exhibited much stronger freezing responses. One-way ANOVA indicates no significant difference in immediate freezing between wild-type and transgenic mice, a significant group difference, however, is found when tested 1 hr $[F(2,24) = 5.062, p < 0.05]$, 1

10   day $[F(2,25) = 5.223, p < 0.05]$, and 10 days $[F(1.18)=4.576, p<0.05]$. Further *post hoc* analysis reveals the significant difference between wild-type and either transgenic line $(p < 0.05, \text{repsectively})$, but not between these two transgenic lines.

15   Since the transgene is also abundantly expressed in the amygdala and the cortex, we then examined the cued fear conditioning. One-way ANOVA indicated that freezing in response to the tone was also significantly elevated in transgenic mice than that in

20   controls when tested at 1 hr $[F(2,24) = 4.672, p < 0.05]$, 1-day $[F(2,25) = 5.518, p < 0.01]$, 10-days $[F(1.18)=6.498, p<0.05]$ after training (Fig. 5d-f). A *post hoc* analysis shows the significant difference between wild-type and either transgenic line $(p<0.05,$

25   respectively). The enhanced contextual and cued fear memories in transgenic mice were not due to an altered nociceptive responses since the minimal amount of current required to elicit three stereotypical behaviors: flinching/running, jumping, and vocalizing were similar

30   between wild-type and transgenic mice.

         We then conducted two additional experiments to measure emotional learning using the fear extinction paradigm (Falls et al., *J. Neurosci.* **12**, 854-863, 1992). It is known if the animals were repeatedly exposed to the

35   context or the CS (tone) without the presence of US

(shock), the context or CS will lose its ability to produce the fearing responses. The reduction in conditioned fear is referred as fear extinction and is an NMDA-dependent process (Falls et al., 1992, *supra*). It is thought to involve the formation of a new memory rather than the passive decay or erasure of the original memory because the original associations remain intact following extinction. We examined the fear extinction using a 5-extinction trial paradigm. When we measured the initial fear response 24 hr after training, again we observed much stronger fear responses in transgenic mice than that of controls in either contextual or cued fear conditioning (Fig. 6). Remarkably, transgenic mice exhibited much less freezing during subsequent exposures to either the context or the tone than that of wild-type mice (Fig. 6a,b). Two-way ANOVA indicated that while both wild-type and transgenic mice decreased their freezing responses to contextual extinction [$F(4,80) = 86.247$, $p < 0.001$] or cued extinction [$F(4,80) = 78.415$, $p < 0.001$], a significant group difference existed between transgenic and wild-type mice in contextual extinction [$F(2,20) = 8.595$, $p < 0.01$] or _n cued extinction was observed [$F(2,20) = 7.778$, $p < 0.01$]. A *post hoc* analysis revealed a significant difference in the freezing responses between wild-type and transgenic mice at the second or third extinction trial in either contextual conditioning ($p < 0.05$, respectively) or cued conditioning ($p < 0.05$, respectively). The similar faster fear extinction was also observed if the experiments were conducted 1 hr after fear conditioning. Therefore, transgenic mice are quicker to learn to disassociate the previous paired event.

Finally, we tested spatial learning in transgenic mice using the hidden-platform water maze, which requires the activation of NMDA receptors in the

hippocampus (Tsien et al., 1996, *supra*; Morris et al., *Nature* **24**, 681-3, 1982). As shown in Fig. 7, the latency to escape to the platform in both wild-type and transgenic mice decreased following the training

5   sessions. However, a significant group difference was observed throughout sessions [$F(1,26) = 9.655$, $p < 0.01$], indicating that spatial learning in transgenic mice was faster than that in wild-type mice. Moreover, a *post hoc* analysis reveals a significant difference at third

10 session ($p < 0.05$), confirming a better learning in transgenic mice. In addition, the enhanced spatial learning in transgenic mice was also evident in the first transfer test conducted after the third training session. In comparison with that of controls, transgenic mice

15 already exhibited clear preference for the targeted quadrant in which the platform was previously located ($p < 0.05$; Student's *t* tests) (Fig. 7b). With additional training, control mice indeed showed the same level of preference in comparison with transgenic mice as measured

20 by either escape latency or place preference in the second transfer test after the last (6th) session. Therefore, these results have demonstrated that these transgenic mice were able to outperform their wild-type littermates in this spatial task.

25

        The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims.

We claim:

1. A method for improving learning or memory
in a subject, which comprises modifying NMDA receptors in
5   neural synapses of the subject's brain, such that NMDA
receptor function is increased by at least 15% as
compared with an equivalent unmodified subject, the
modification resulting in improved learning or memory in
the subject.
10

2. The method of claim 1, comprising
modulating NMDA receptor function in the subject's brain
with a chemical compound.

15 3. The method of claim 1, comprising
increasing a ratio of NR2B to NR2A subunits in the NMDA
receptors of the subject's brain.

4. The method of claim 3, comprising treating
20 the subject to increase production or decrease
degradation of NR2B receptor subunits in the subject's
brain, resulting in an elevated amount of NR2B subunit as
compared with the amount produced by an equivalent, but
untreated subject.
25

5. The method of claim 4, wherein the
treatment results in the presence of at least twenty
percent more NR2B subunit in the treated subject, as
compared with an equivalent, but untreated, subject.
30

6. The method of claim 4, wherein the treating
comprises genetically altering the subject with an
exogenous nucleic acid molecule that encodes NR2B,
constructed so as to produce NR2B in the brain of the
35 subject.

7. The method of claim 6, wherein the genetic alteration is inheritable.

8. The method of claim 6, wherein the genetic alteration is not inheritable.

9. The method of claim 1, wherein the subject is an adult.

10. The method of claim 1, wherein the subject is a juvenile.

11. The method of claim 7, wherein the subject is an embryo.

12. The method of claim 1, wherein the subject is a mammal.

13. The method of claim 12, wherein the mammal is a mouse.

14. The method of claim 12, wherein the mammal is a human.

15. A method of treating a neurodegenerative disorder affecting learning or memory in a patient in need of such treatment, which comprises modifying NMDA receptors in neural synapses of the patient's brain, such that the NMDA receptor function is increased by at least 15% as compared with an equivalent unmodified patient, the modification resulting in improved learning or memory in the patient.

16.   The method of claim 15, comprising
enhancing NMDA receptor function in the brain with a
chemical compound.

5   17.   The method of claim 15, comprising
increasing a ratio of NR2B-containing NMDA receptors to
NR2A-containing NMDA receptors in the patient's brain.

18.   The method of claim 17, comprising
10   stimulating production or inhibiting degradation of NR2B
receptor subunits in the patient's brain, resulting in an
increased amount of NR2B subunit as compared with the
amount produced by an equivalent, but untreated patient.

15   19.   The method of claim 18, wherein the
treatment results in at least twenty percent more NR2B
subunit than found in an equivalent, but untreated
patient.

20   20.   The method of claim 18, wherein the
stimulation of production of NR2B subunits is
accomplished by genetically altering the patient with an
exogenous nucleic acid molecule that encodes NR2B,
constructed so as to produce NR2B in the brain of the
25   patient.

21.   The method of claim 15, wherein the
patient is an adult.

30   22.   The method of claim 15, wherein the
patient is a juvenile.

23.   A genetically altered non-human animal
having enhanced synaptic plasticity and improved learning
35   and memory as compared with an equivalent, but unaltered

animal, the genetic alteration resulting in a
modification of NMDA receptors in neural synapses of the
patient's brain, such that the NMDA receptor function is
increased by at least 15% as compared with an equivalent
5 unaltered animal.

        24. The genetically modified animal of claim
23, wherein the animal over-expresses an endogenous gene
encoding NR2B.
10

        25. The genetically modified animal of claim
23, wherein the animal expresses a transgene encoding
NR2B.

15   26. The transgenic animal of claim 23,
selected from the group consisting of mouse, rat, cat,
dog, dolphin and non-human primate.

        27. The transgenic animal of claim 23, wherein
20 the genetic alteration is inheritable.

        28. A method of identifying compounds that
enhance learning and memory in a subject by increasing
expression of NR2B genes in the subject, which comprises
25 providing a chimeric DNA construct comprising an NR2B
promoter operably linked to a reporter gene, contacting
the chimeric DNA construct with a test compound suspected
of up-regulating the NR2B promoter, and measuring
expression of the reporter gene, an increase in the
30 expression being indicative that the test compound
enhances learning and memory in the subject by increasing
expression of NR2B genes in the subject.

        29. An method for identifying compounds that
35 enhance learning and memory in a subject by affecting

expression of NR2B or activity of NMDA receptors, which comprises:

     a) providing a pair of equivalent cells, one being transgenic and expressing an exogenous nucleic acid molecule encoding NR2B, and the other being non-transgenic for expression of an exogenous nucleic acid molecule encoding NR2B;

     b) treating the non-transgenic cell with a test compound suspected to affect the expression NR2B or activity of the NMDA receptors;

     c) comparing NMDA receptor function of the treated, non-transgenic cell with NMDA receptor function of the transgenic cell and, optionally, NMDA receptor function of an untreated, non-transgenic cell, a change in NMDA receptor function in the treated, non-transgenic cell that comprises the same features of NMDA receptor function exhibited in the transgenic cell being indicative that the test compound enhances learning and memory in a subject by affecting expression of NR2B or activity of NMDA receptors.

     30. The method of claim 29, wherein the cells are disposed within a tissue.

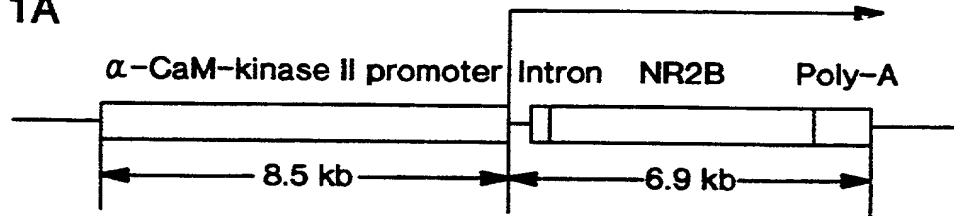   31. The method of claim 30, wherein the tissue is disposed within a living animal.

     32. The method of claim 30, wherein the NMDA receptor function is measured electrophysiologically.

     33. The method of claim 32, wherein the NMDA receptor function is measured by measuring peak amplitude or channel decay time of NMDA receptors.

- 50 -

34.   The method of claim 31, wherein the NMDA receptor function is measured using behavioral tests of learning and memory.

5   35.   An *in vivo* assay for identifying compounds that enhance function of NMDA receptors in a subject, which comprises:
         a) providing a pair of animals;
         b) treating one of the animals with a test
10   compound suspected of enhancing NMDA receptor function; and
         c) directly or indirectly measuring a change in NMDA function in the treated animal as compared with the untreated animal, a change being indicative that the test
15   compound affects NMDA receptor function in a subject.

36.   The method of claim 35, wherein the animals are the genetically altered animals of claim 23.

20   37.   The method of claim 35, wherein the NMDA receptor function is measured electrophysiologically.

38.   The method of claim 35, wherein the NMDA receptor function is measured by measuring peak amplitude
25   or channel decay time of NMDA receptors.

39.   The method of claim 35, wherein the NMDA receptor function is measured using behavioral tests of learning and memory.

30

40.   An *in vitro* assay for identifying compounds that enhance function of NMDA receptors in a subject, which comprises:
         a) providing a pair of cells;
35   b) treating one of the cells with a test

compound suspected of enhancing NMDA receptor function; and

        c) directly or indirectly measuring a change in NMDA function in the treated cell as compared with the untreated cell, a change being indicative that the test compound affects NMDA receptor function in a subject.

        41.   The method of claim 40, wherein the cells are genetically altered so as to possess enhanced NMDA receptor function as compared with equivalent, but unaltered cells.

        42.   The method of claim 40, wherein the NMDA receptor function is measured electrophysiologically.

        43.   The method of claim 42, wherein the NMDA receptor function is measured by measuring peak amplitude or channel decay time of NMDA receptors.

        44.   A method of identifying genes and gene products that affect NMDA receptor-mediated learning and memory in a subject, which comprises:

        a) providing a pair of equivalent animals, one being transgenic and expressing an exogenous nucleic acid molecule encoding NR2B, and the other being non-transgenic for expression of an exogenous nucleic acid molecule encoding NR2B;

        b) comparing profiles of gene expression or protein modification in the transgenic and non-transgenic animals;

        c) isolating one or more genes or gene products whose expression is altered or modified in the transgenic animal; and

        d) identifying the one or more genes or gene products.

45. A method of identifying genes and gene products that affect NMDA receptor-mediated learning and memory in a subject, which comprises:

a) providing cells containing NMDA receptors;

b) stimulating the NMDA receptors in a sample of the cells;

c) comparing profiles of gene expression or protein modification in the cell sample having stimulated NMDA receptors with an equivalent cell sample wherein the NMDA receptors are unstimulated;

d) isolating one or more genes or gene products whose expression is altered in the cells having stimulated NMDA receptors; and

d) identifying the one or more genes or gene products.

46. The method of claim 45, wherein the NMDA receptors are stimulated by treatment with a chemical compound.

47. The method of claim 45, wherein the NMDA receptors are stimulated electrically.

48. The method of claim 45, wherein the cells are disposed within a tissue.

49. The method of claim 45, wherein the cells are disposed within a living organism.

FIG. 1A

α−CaM−kinase II promoter | Intron   NR2B   Poly−A

←————— 8.5 kb —————→←————— 6.9 kb —————→

FIG. 1B
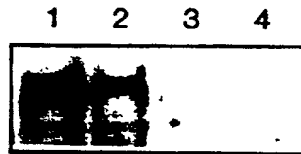
FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1F

FIG. 1G

FIG. 2A

FIG. 2B

FIG. 2C

Day 10

NR2B = 202 ms

WT = 201 ms

Day 14

WT = 238 ms

NR2B = 188 ms

Day 18

NR2B = 273 ms

WT = 122 ms

20 pA

100 ms
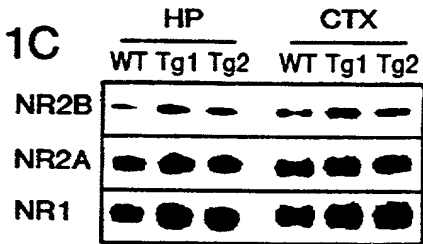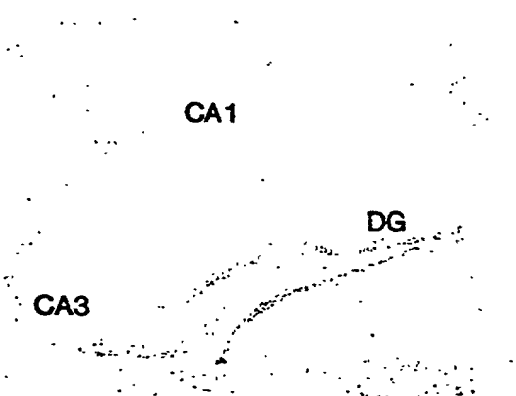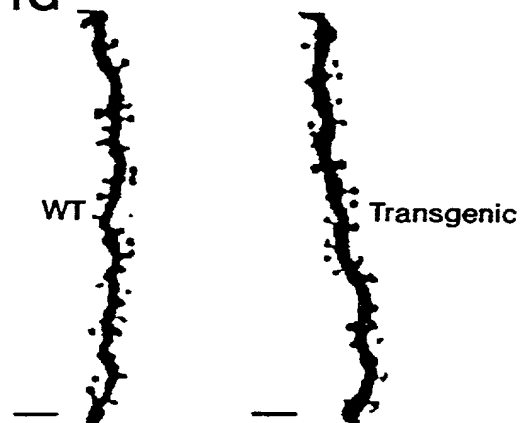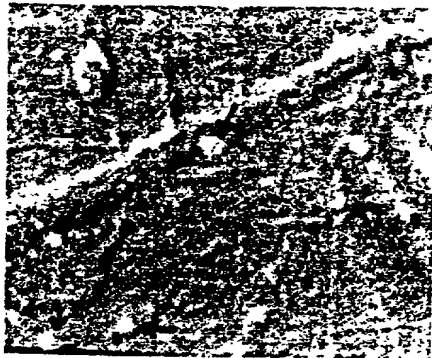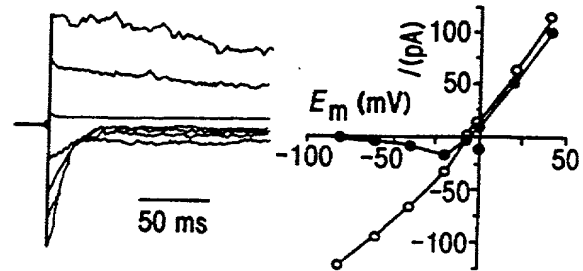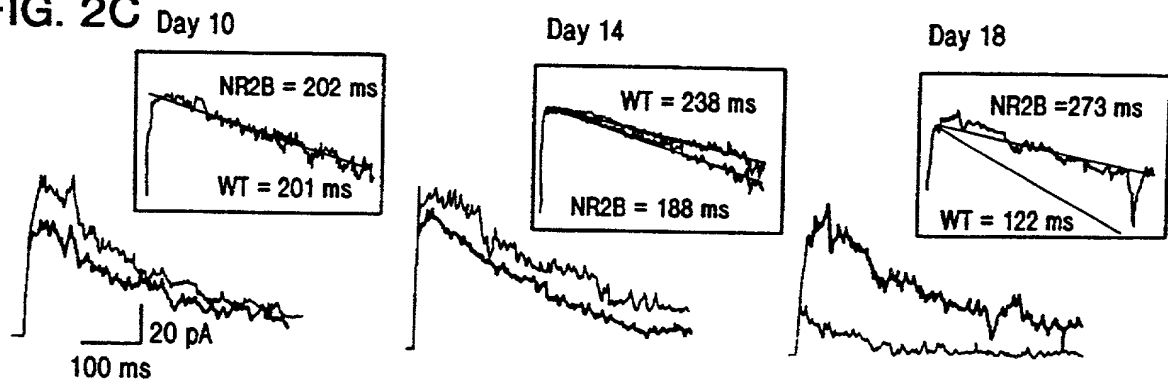
FIG. 2D
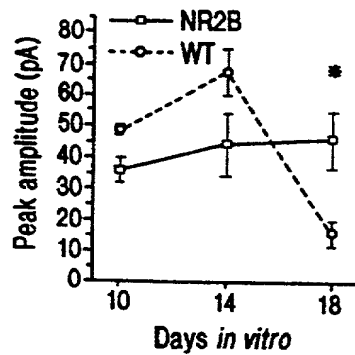
FIG. 2E
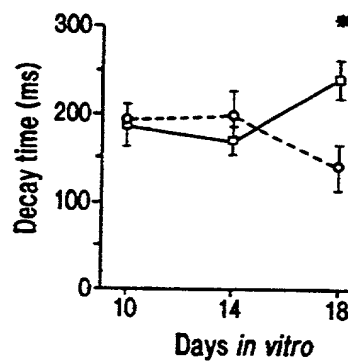
FIG. 2F
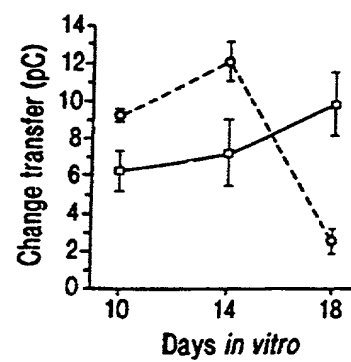
FIG. 3A
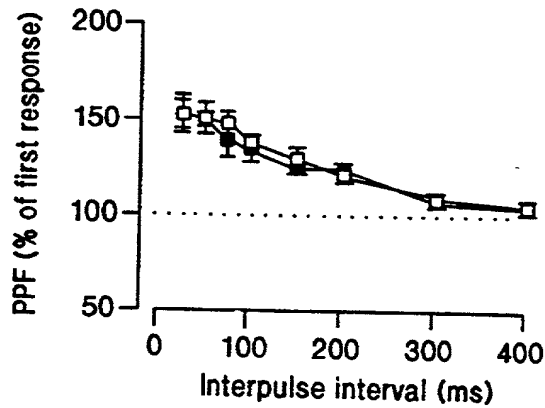
FIG. 3B
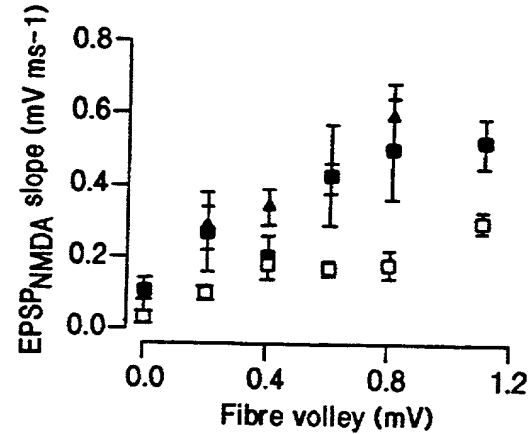
FIG. 3C
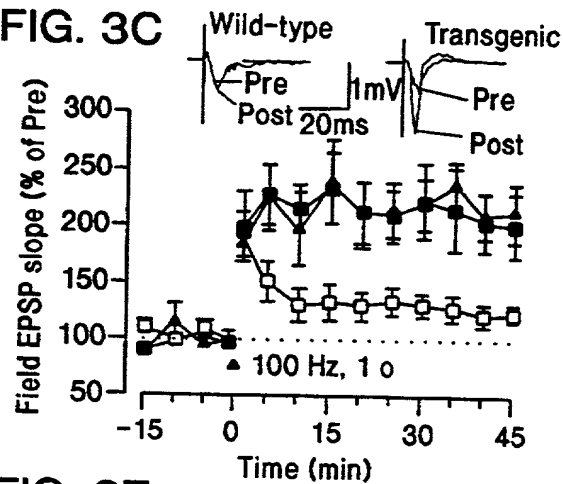
FIG. 3D
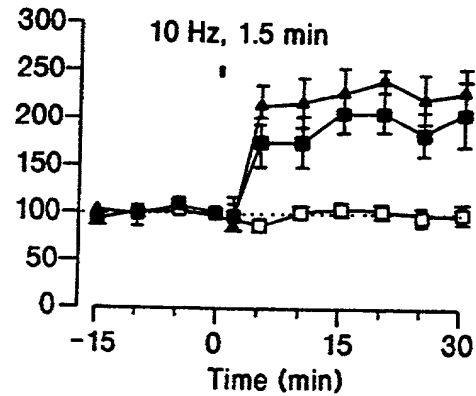
FIG. 3E
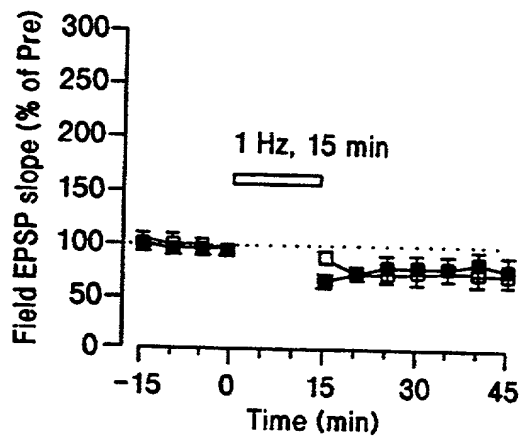
FIG. 3F
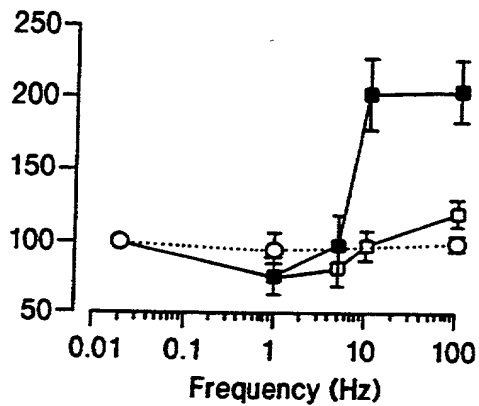
FIG. 4A
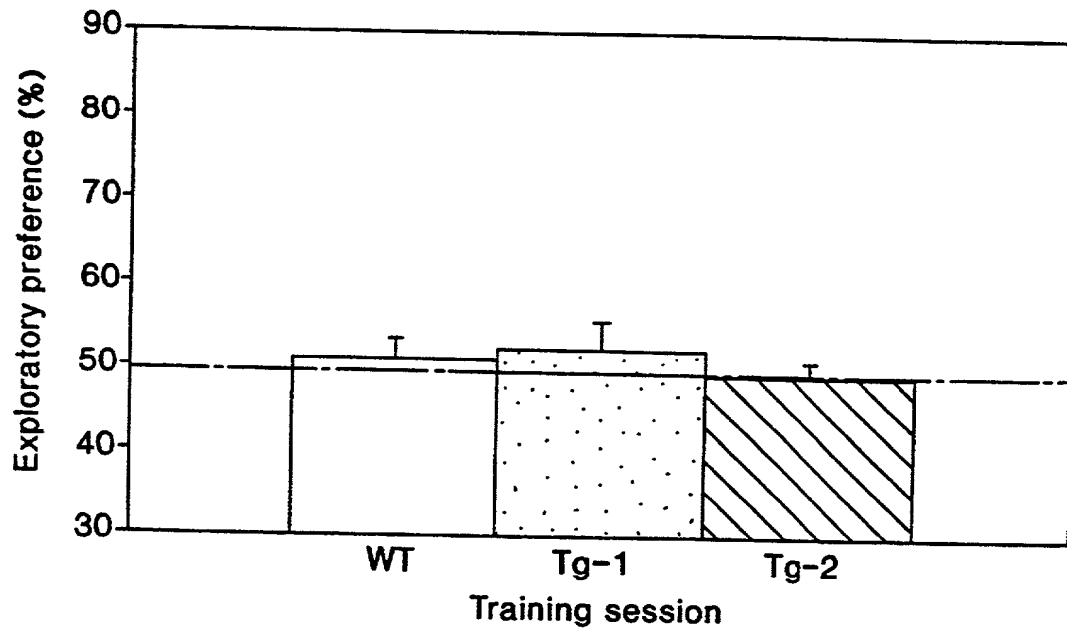
FIG. 4B
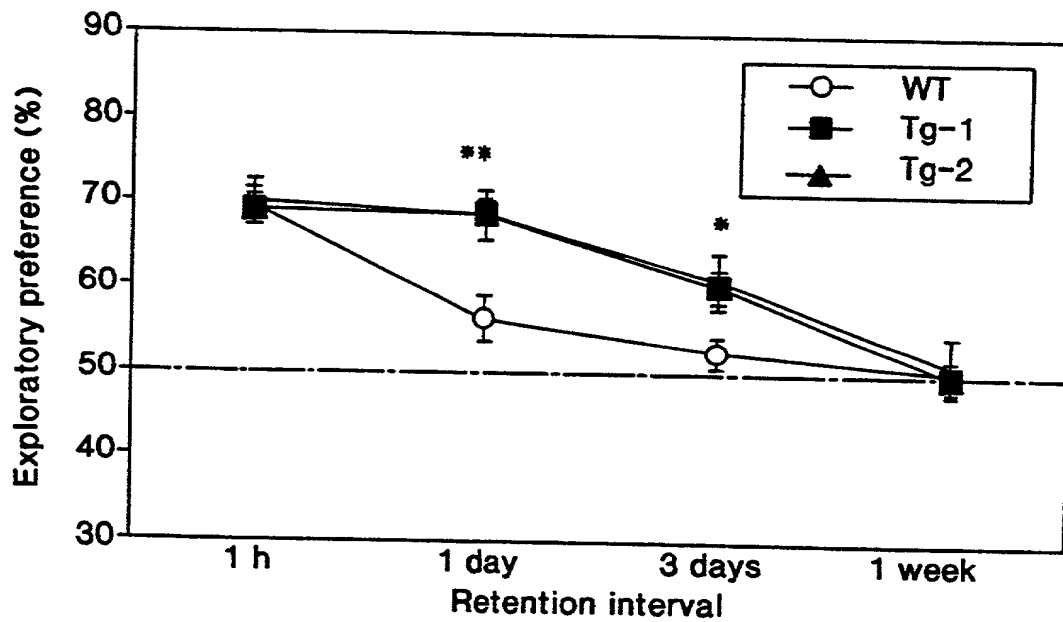
FIG. 5A
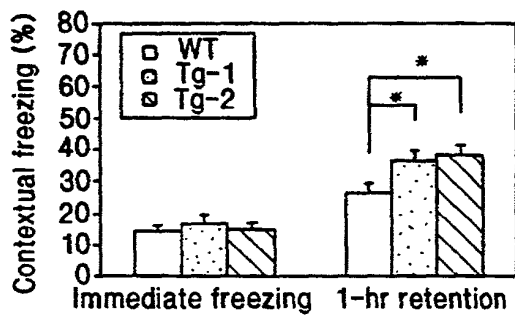
FIG. 5D

FIG. 5B

FIG. 5E
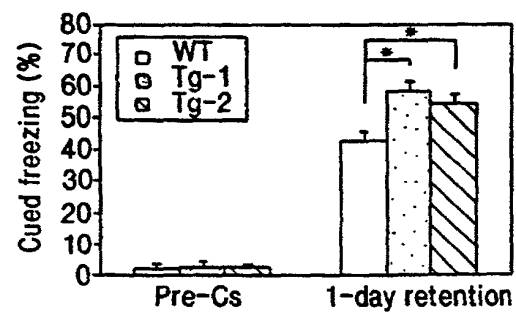
FIG. 5C
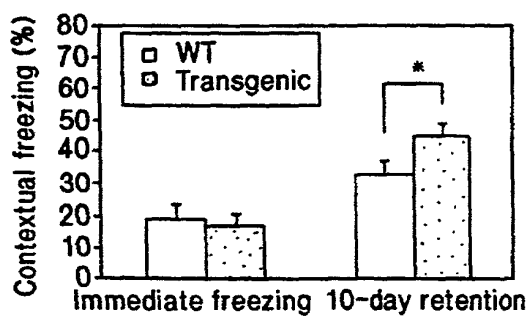
FIG. 5F
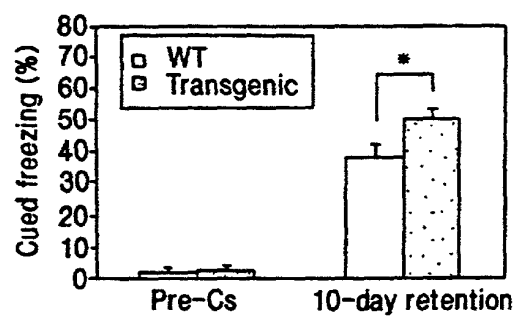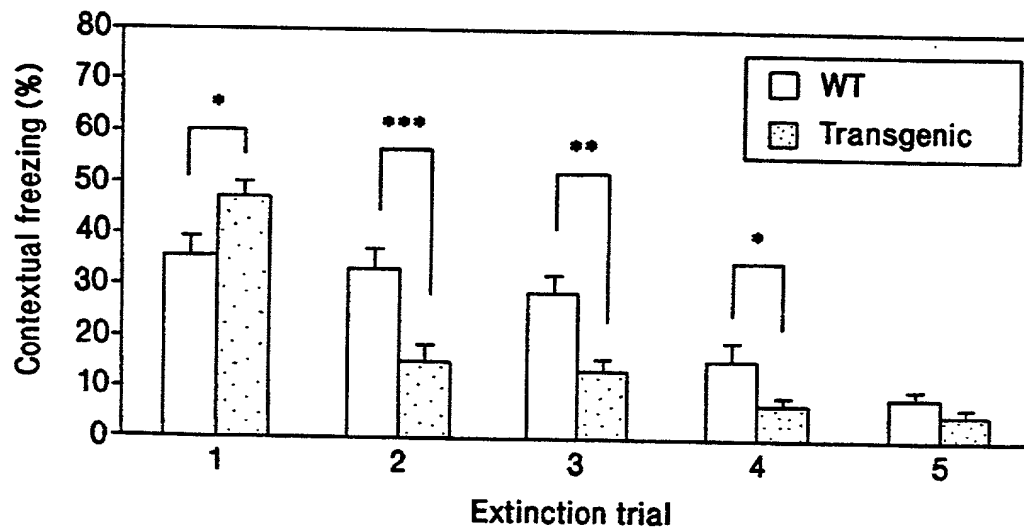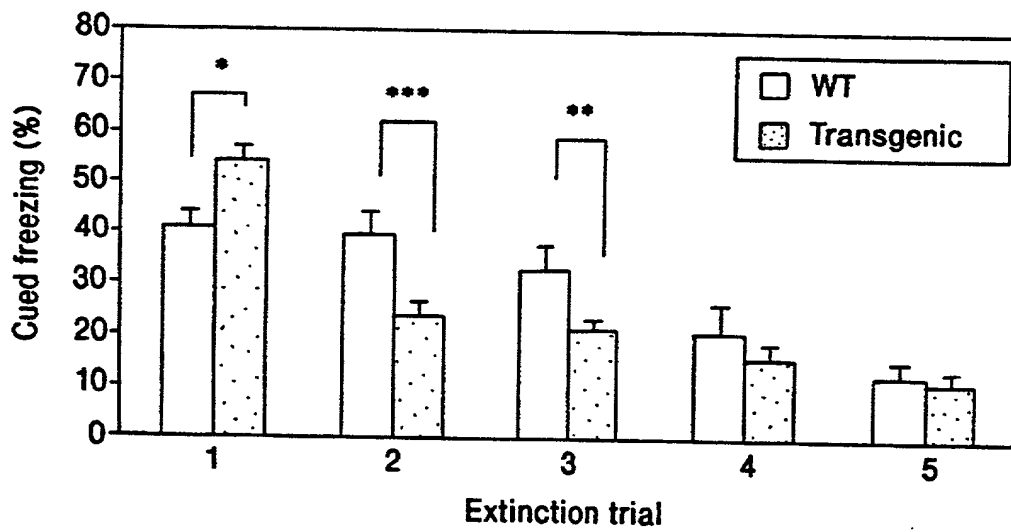
FIG. 6A

FIG. 6B

FIG. 7A

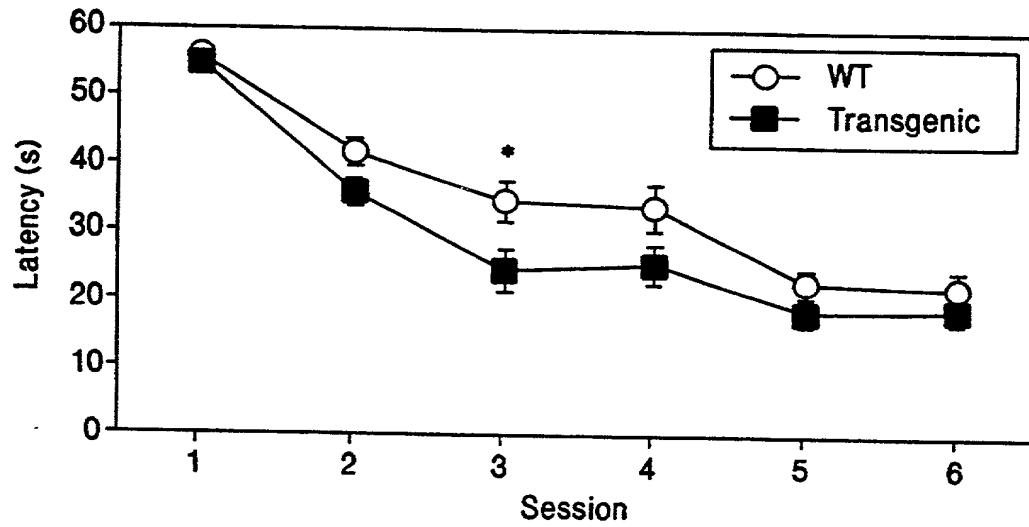## FIG. 7B

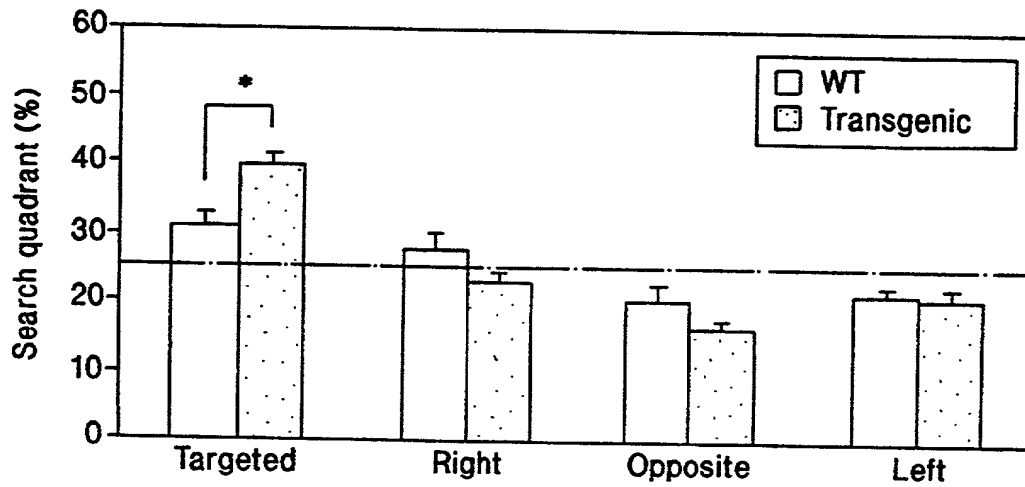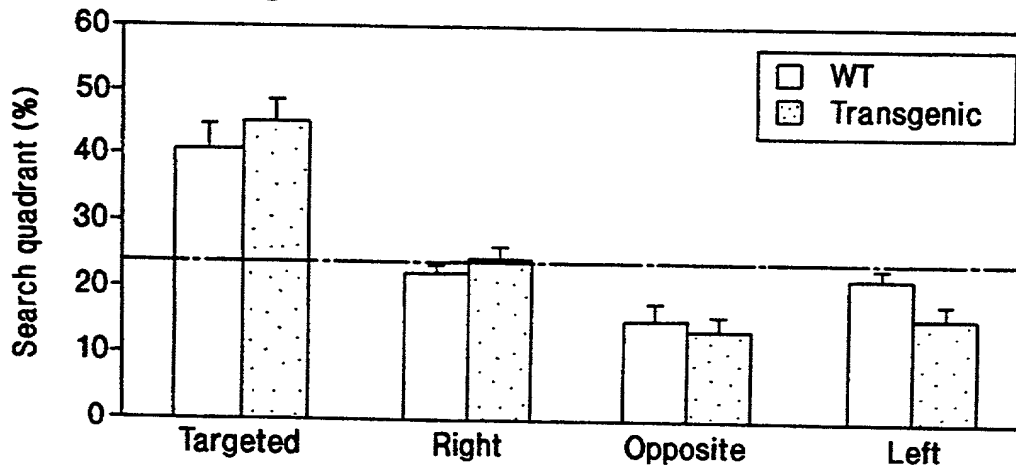## FIG. 7C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Joe Z. TSIEN   **Group Art Unit:** not yet known

Intl. Appln. No.: PCT/US00/12528   **Examiner:** not yet assigned

Intl. Filing Date: 08 May 2000

For: COMPOSITIONS AND METHODS FOR
         IMPROVING LEARNING AND
         MEMORY

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a

☒   Utility Patent   ☐   Design Patent

is sought on the invention, whose title appears above, the specification of which:

☐   is attached hereto.

☒   was filed on __08 May 2000__ as International Application
     No. __PCT/US00/12528__ .

☐   said application having been amended on _____ .

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR §

1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Priority Claimed (If X'd)	**Country**	**Serial Number**	**Date Filed**
☐	_____	_____	_____
☐	_____	_____	_____
☐	_____	_____	_____
☐	_____	_____	_____

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	**Date Filed**	**Patented/Pending/Abandoned**
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Serial Number	Date Filed
60/133,371	10 May 1999

I hereby appoint the following persons of the firm of **WOODCOCK WASHBURN LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103 as attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

John W. Caldwell   Reg. No. 28,937

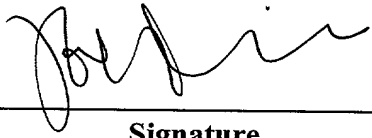Janet E. Reed   Reg. No. 36,252

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Telephone No.: **(215) 568-3100**
Facsimile No.: **(215) 568-3439**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name: Joe Z. TSIEN	Signature
Mailing Address: Princeton, NJ 08544 United States of America	Date of Signature: _Dec. 12/ 2001_
City/State of Actual Residence: Princeton, New Jersey	Citizenship: _____ United States _____

DOCKET NO. : PU-0082

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Joe Z. TSIEN

Serial No.: 10/009,228   **Group Art Unit:** not yet known

Intl. Application No.: PCT/US00/12528

Intl. Filing Date: 08 May 2000   **Examiner:** not yet assigned

For: COMPOSITIONS AND METHODS FOR IMPROVING LEARNING AND MEMORY

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

ASSOCIATE POWER OF ATTORNEY

The undersigned, of the firm WOODCOCK WASHBURN LLP, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, Attorney and/or Agents for Applicant(s), hereby appoints the following:

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Doreen Yatko Trujillo	Registration No. 35,719	Frank T. Carroll	Registration No. 42,392
Barbara L. Mullin	Registration No. 38,250	Thomas E. Watson	Registration No. 43,243
Michael P. Straher	Registration No. 38,325	Eric H. Vance	Registration No. 47,151
Kevin M. Flannery	Registration No. 35,871	Peter M. Ullman	Registration No. 43,963
David A. Cherry	Registration No. 35,099	Richard B. LeBlanc	Registration No. 39,495
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		George J. Awad	Registration No. 46,528

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Daniel D. Biesterveld	Registration No. 45,898		
Phillip A. Singer	Registration No. 40,176		

his/her associates with full power to prosecute the above-identified application and to transact all business in the Patent Office connected therewith and requests that correspondence continue to be directed to the firm of WOODCOCK WASHBURN LLP at the above address.

Date: 3/12/02

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